

EXHIBIT 42

US 10,632,466 B1

Page 2

Related U.S. Application Data

- No. 14/941,087, filed on Nov. 13, 2015, now Pat. No. 10,071,376, which is a continuation of application No. 12/218,498, filed on Jul. 14, 2008, now Pat. No. 9,186,677, which is a continuation-in-part of application No. 11/985,577, filed on Nov. 14, 2007, now Pat. No. 7,998,708.
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CPC **F16K 99/0044** (2013.01); **F16K 99/0061** (2013.01); **B01L 2200/027** (2013.01); **B01L 2200/10** (2013.01); **B01L 2200/147** (2013.01); **B01L 2200/148** (2013.01); **B01L 2200/16** (2013.01); **B01L 2300/021** (2013.01); **B01L 2300/045** (2013.01); **B01L 2300/06** (2013.01); **B01L 2300/0627** (2013.01); **B01L 2300/0681** (2013.01); **B01L 2300/087** (2013.01); **B01L 2300/0816** (2013.01); **B01L 2300/0832** (2013.01); **B01L 2300/0867** (2013.01); **B01L 2300/0887** (2013.01); **B01L 2300/18** (2013.01); **B01L 2300/1822** (2013.01); **B01L 2300/1827** (2013.01); **B01L 2300/1861** (2013.01); **B01L 2400/0442** (2013.01); **B01L 2400/0481** (2013.01); **B01L 2400/0487** (2013.01); **B01L 2400/0611** (2013.01); **B01L 2400/0677** (2013.01); **B01L 2400/0683** (2013.01); **F16K 2099/0084** (2013.01); **G01N 35/026** (2013.01); **G01N 2035/00881** (2013.01); **G01N 2035/0425** (2013.01); **G01N 2035/0436** (2013.01)

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Page 3

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US 10,632,466 B1

Page 12

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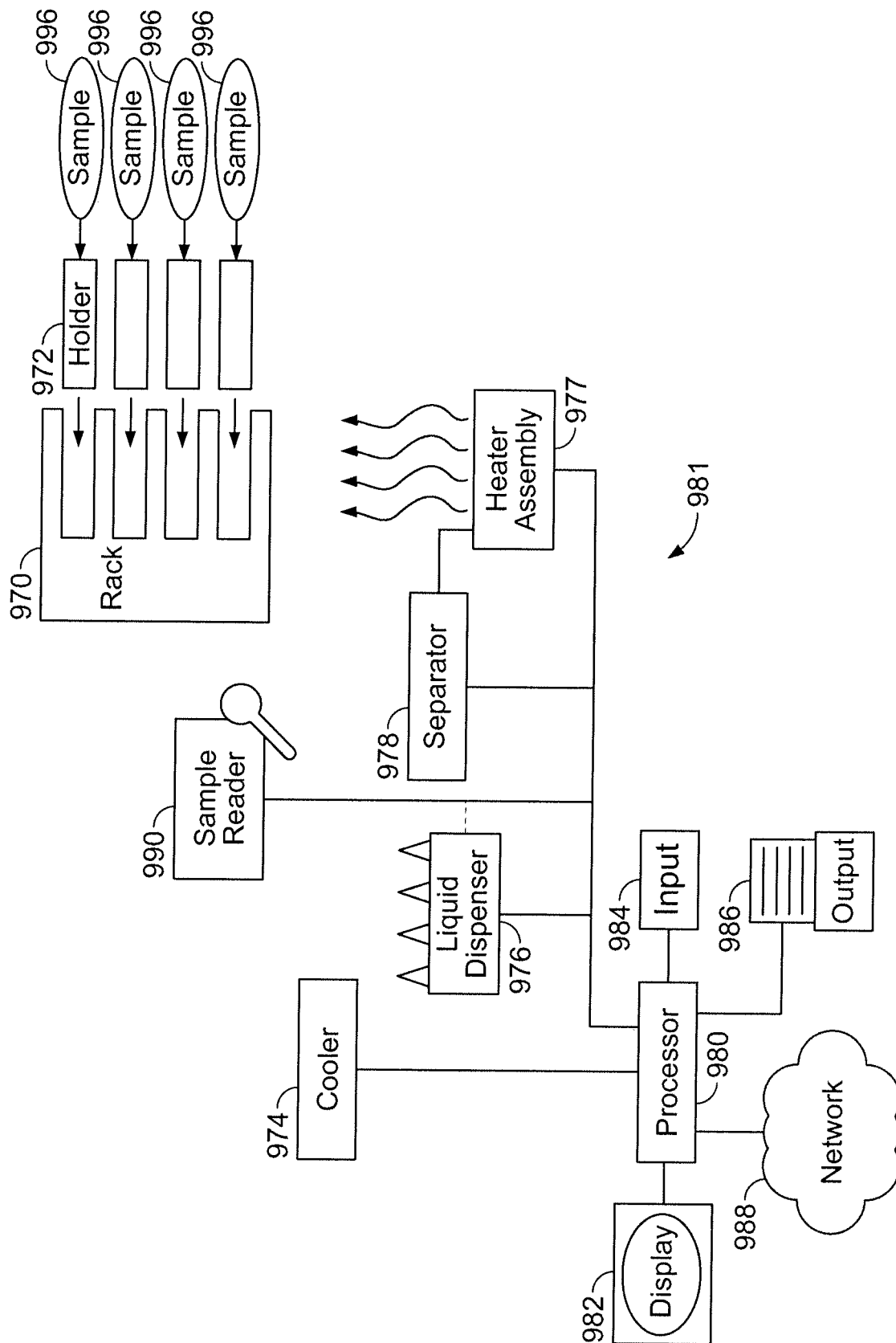


FIG. 1A

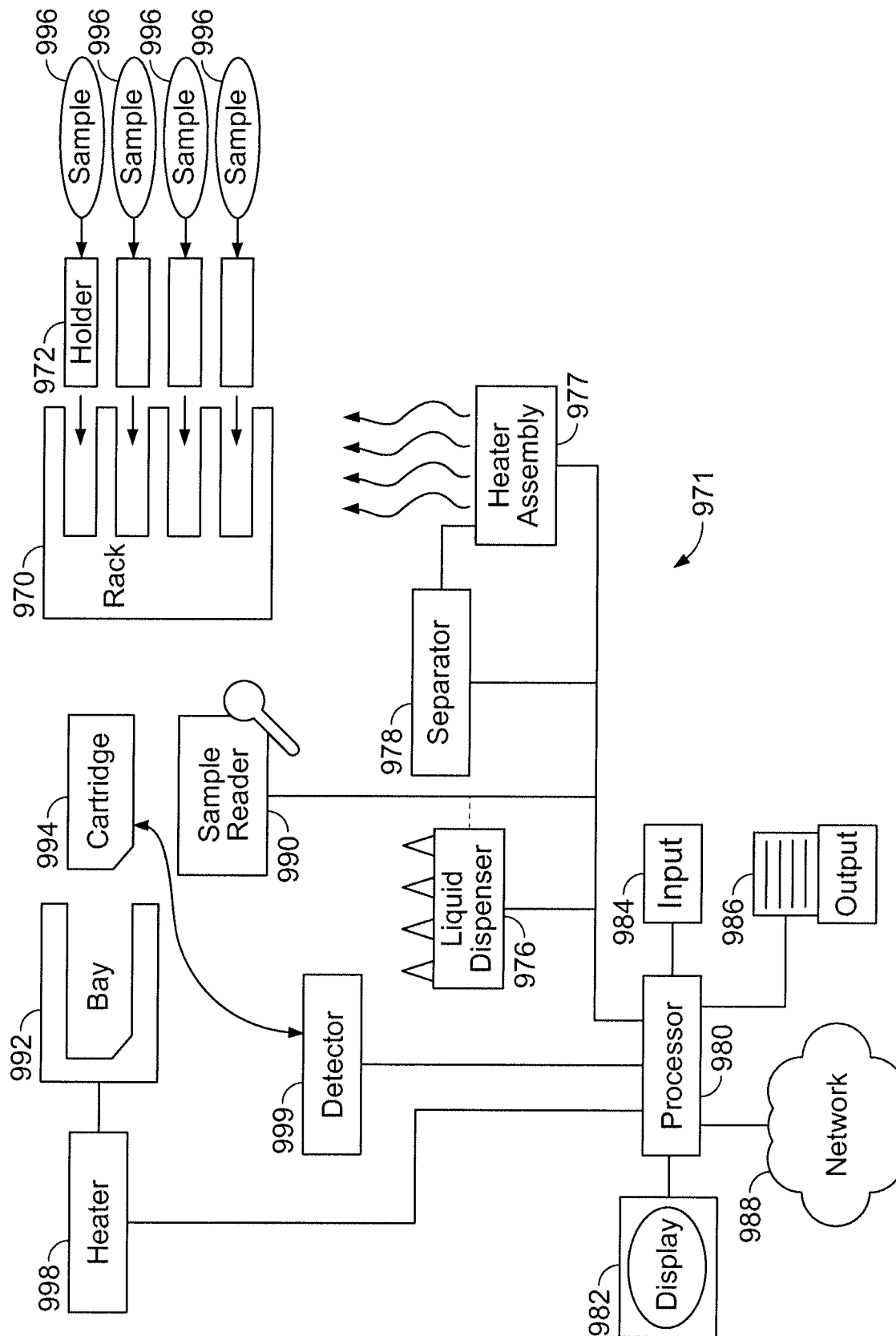


FIG. 1B

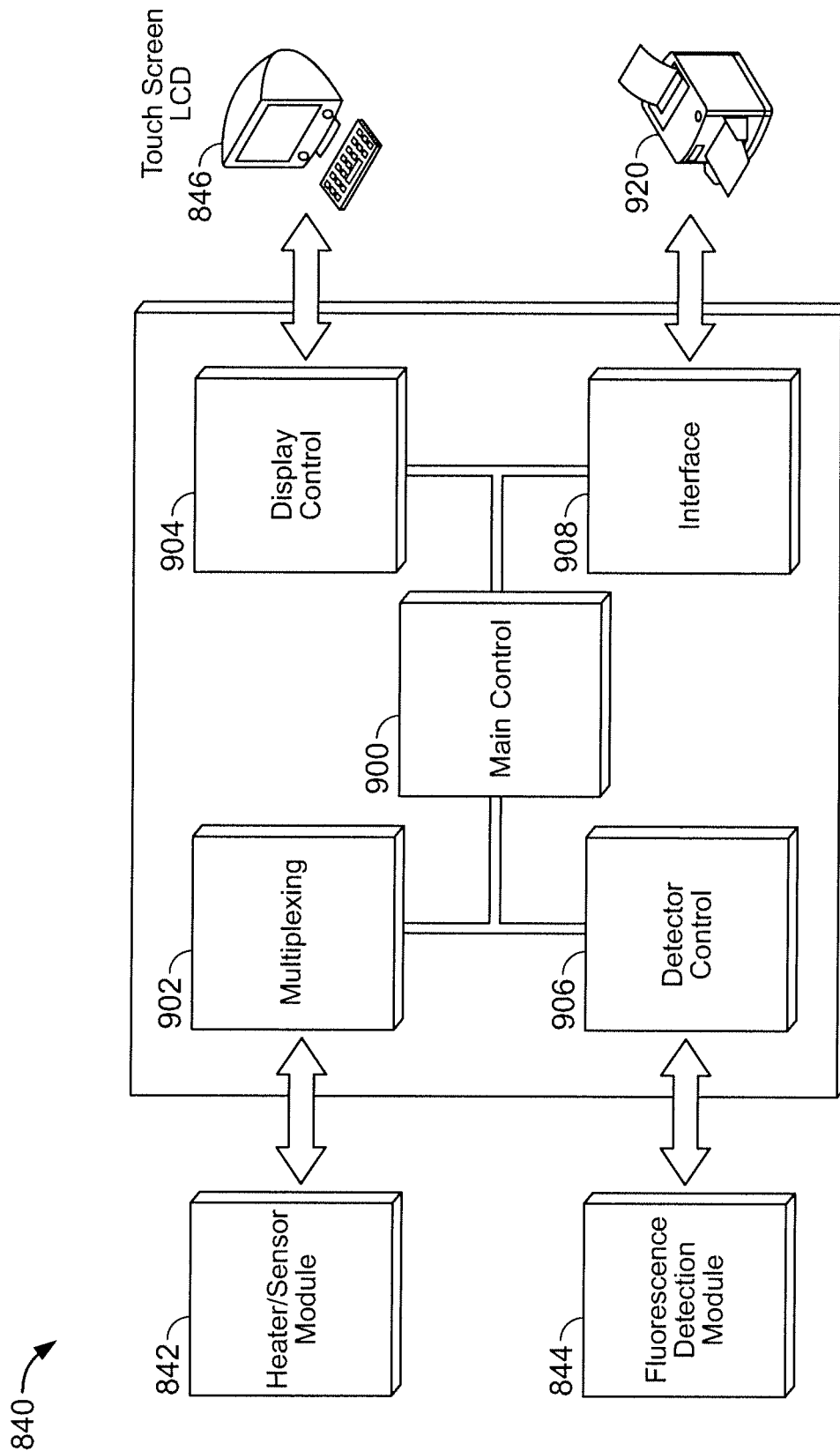


FIG. 2

U.S. Patent

Apr. 28, 2020

Sheet 4 of 121

US 10,632,466 B1

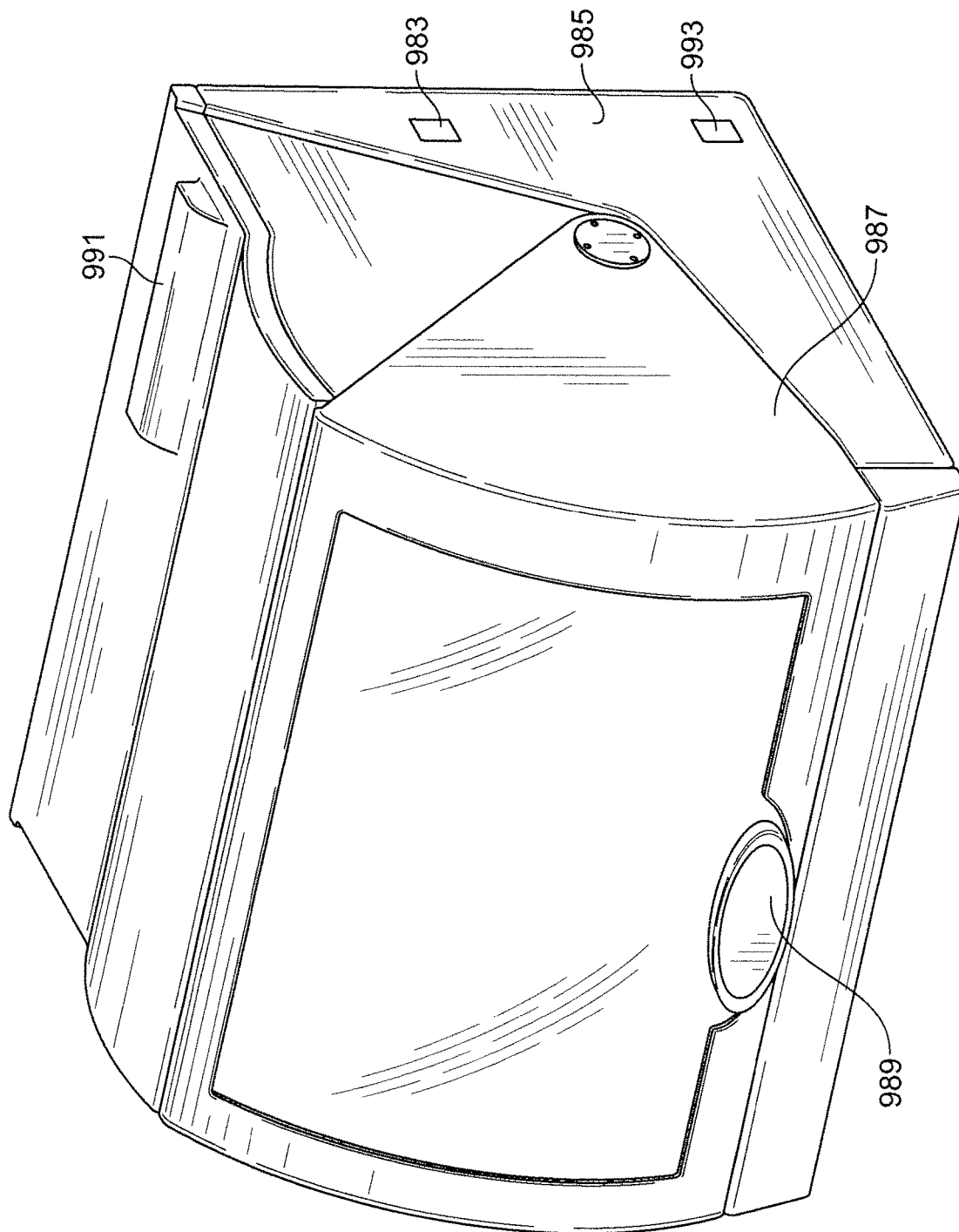


FIG 3A

U.S. Patent

Apr. 28, 2020

Sheet 5 of 121

US 10,632,466 B1

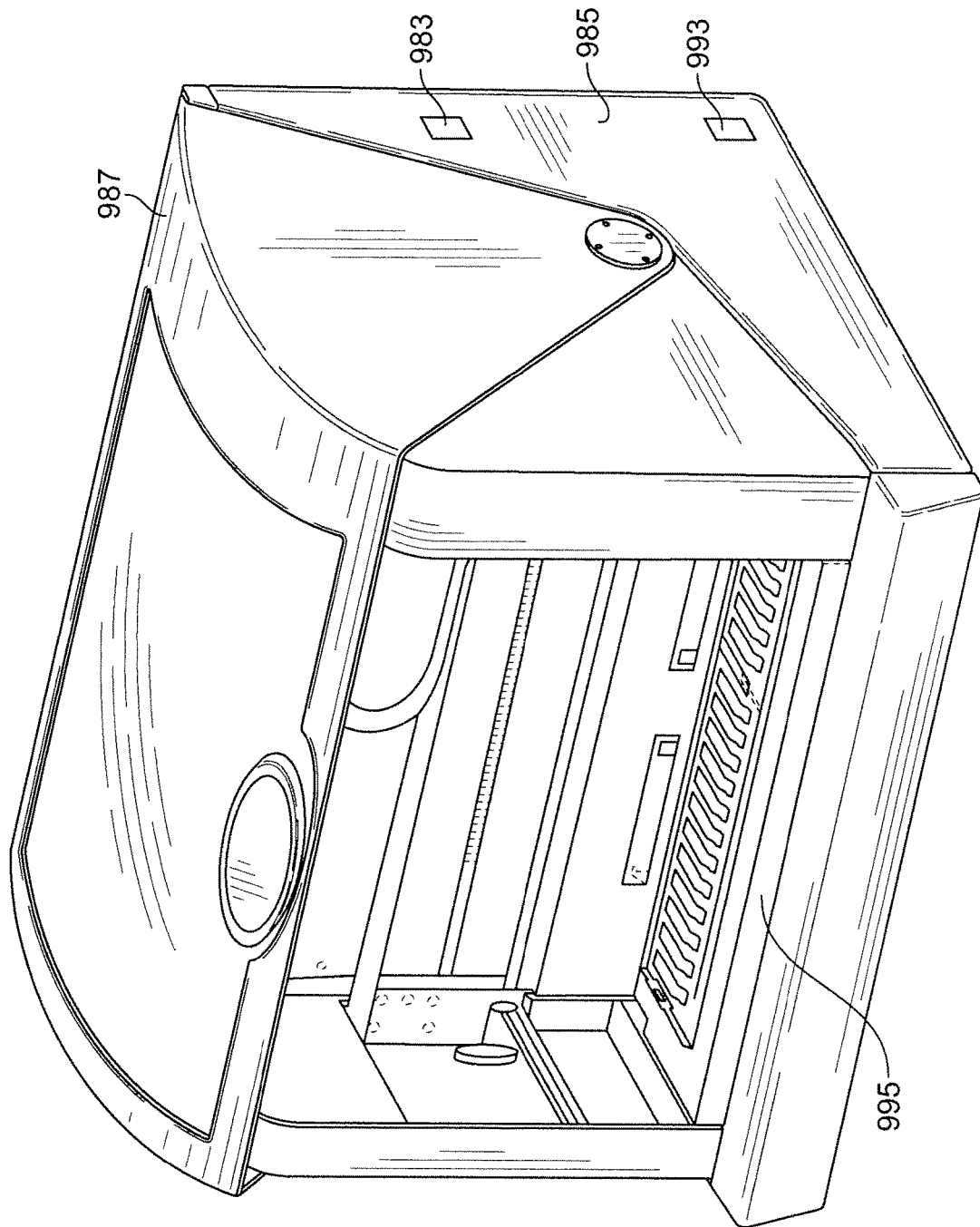


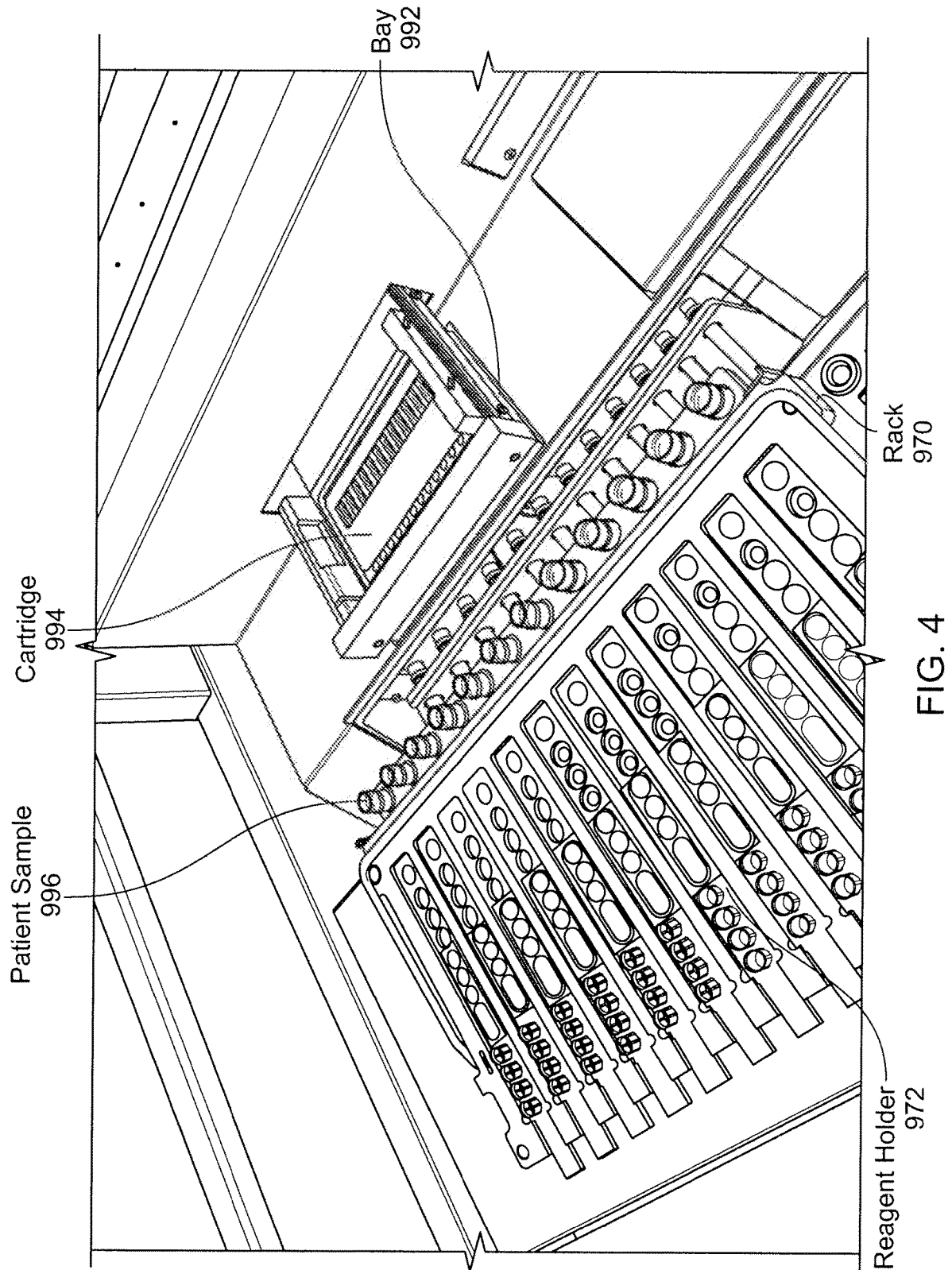
FIG. 3B

U.S. Patent

Apr. 28, 2020

Sheet 6 of 121

US 10,632,466 B1



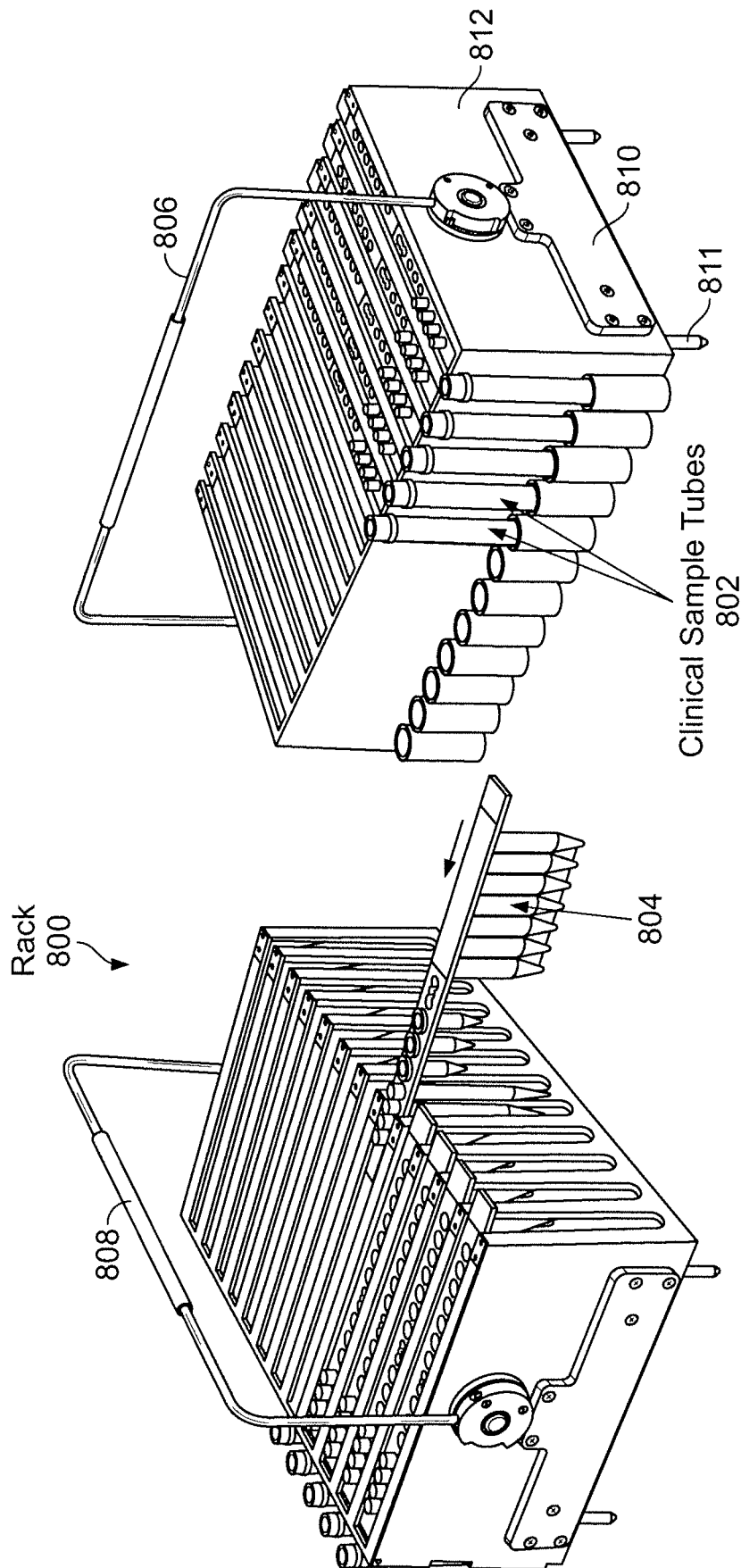


FIG. 5

U.S. Patent

Apr. 28, 2020

Sheet 8 of 121

US 10,632,466 B1

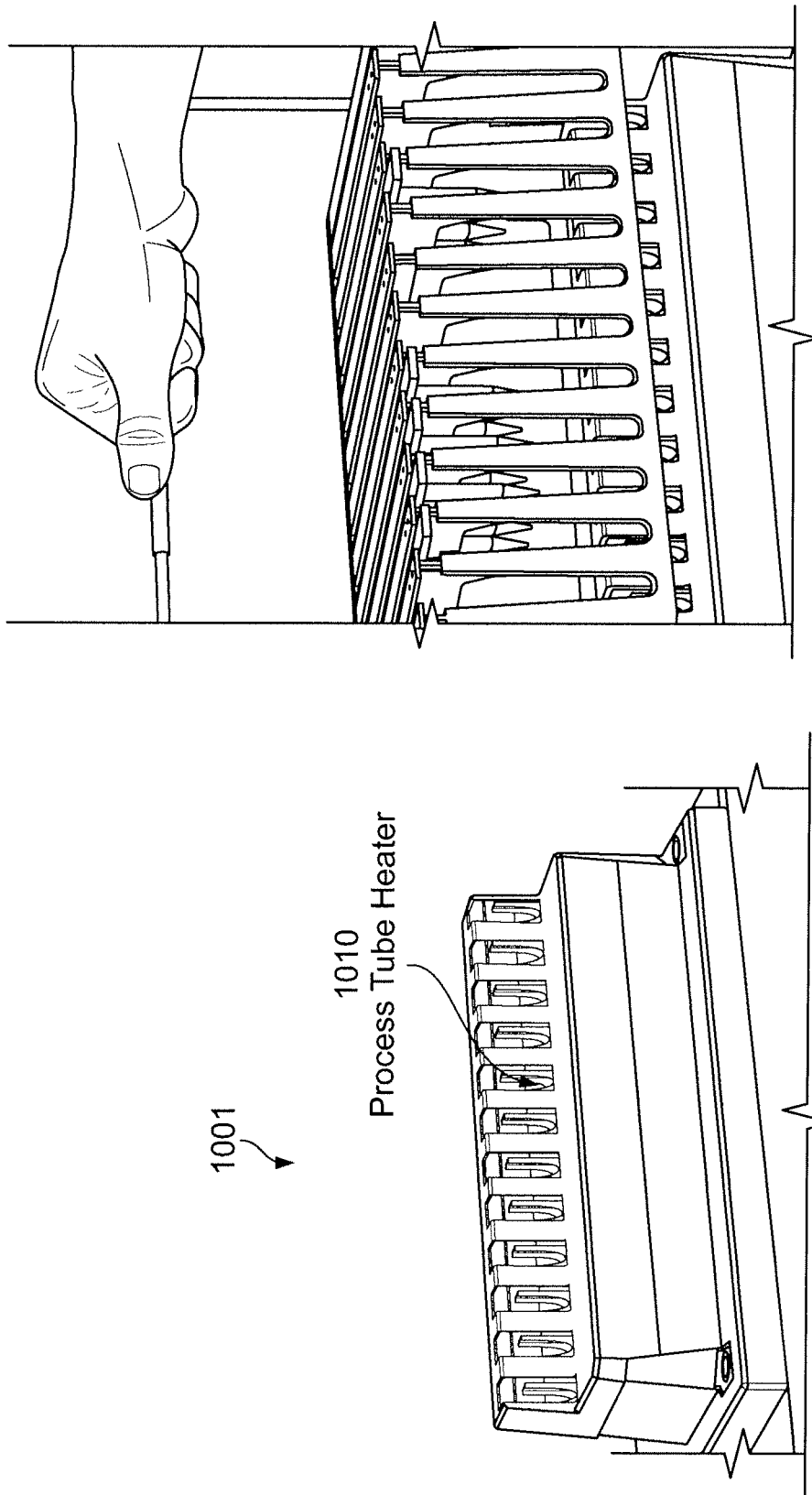
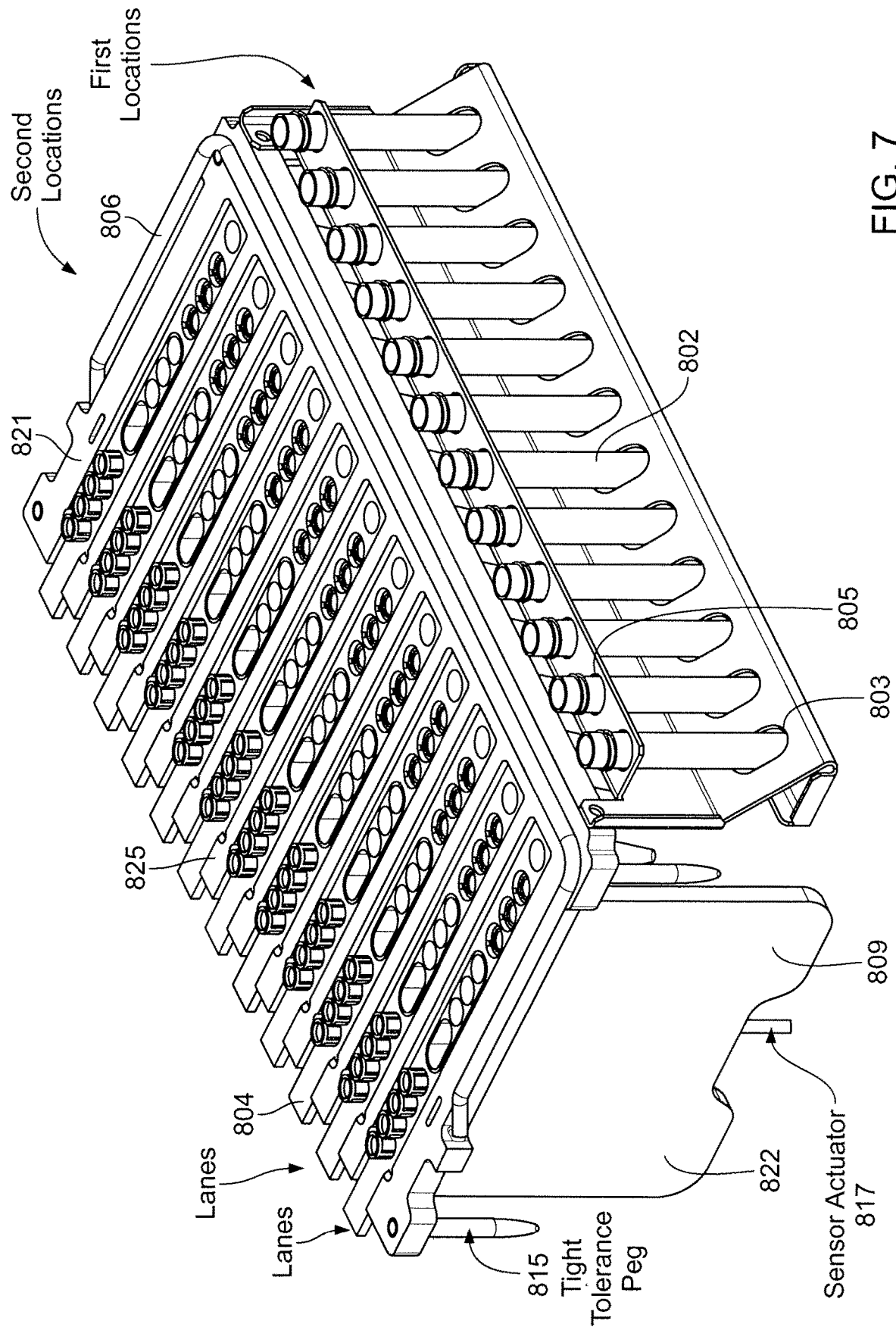


FIG. 6



U.S. Patent

Apr. 28, 2020

Sheet 10 of 121

US 10,632,466 B1

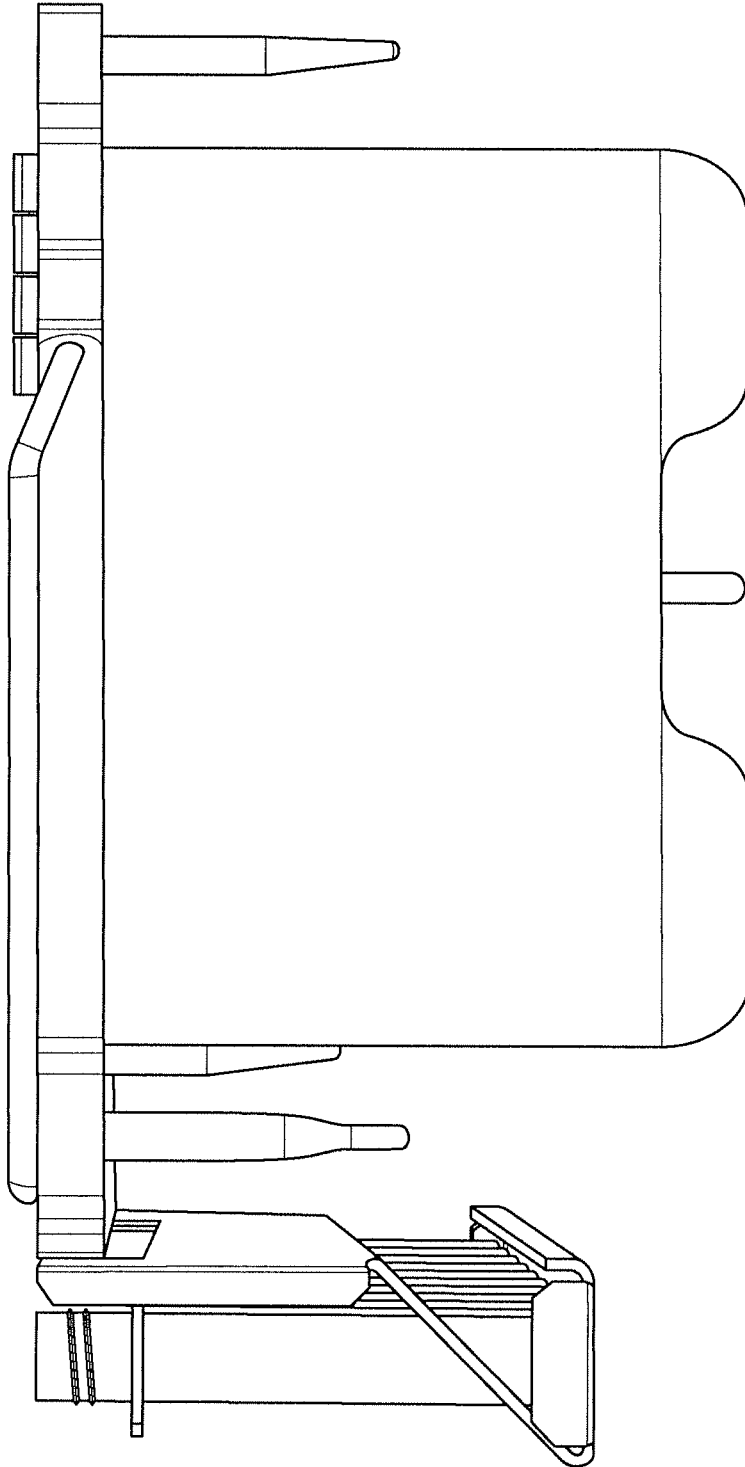


FIG. 8A

U.S. Patent

Apr. 28, 2020

Sheet 11 of 121

US 10,632,466 B1

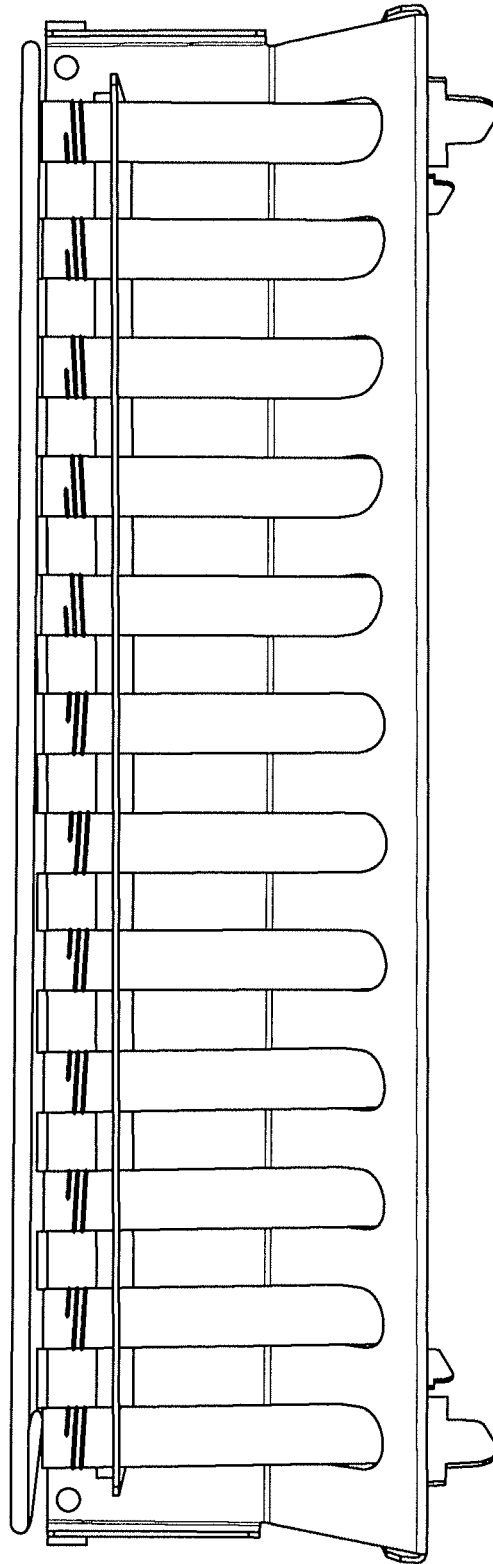


FIG. 8B

U.S. Patent

Apr. 28, 2020

Sheet 12 of 121

US 10,632,466 B1

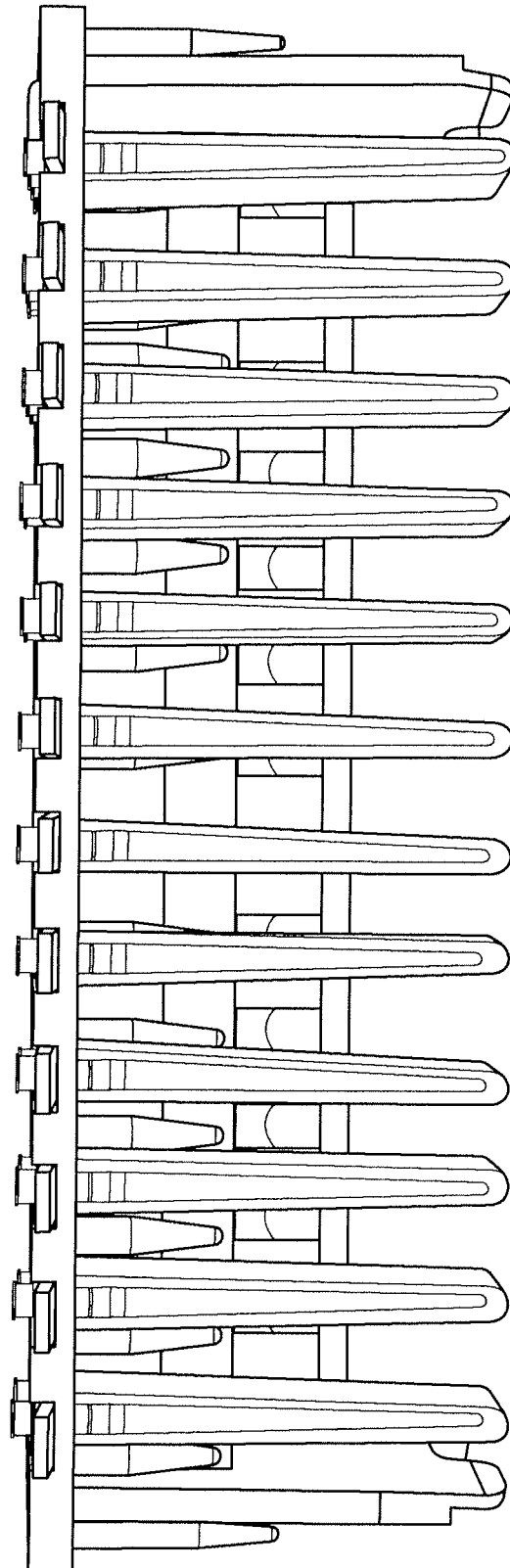


FIG. 8C

U.S. Patent

Apr. 28, 2020

Sheet 13 of 121

US 10,632,466 B1

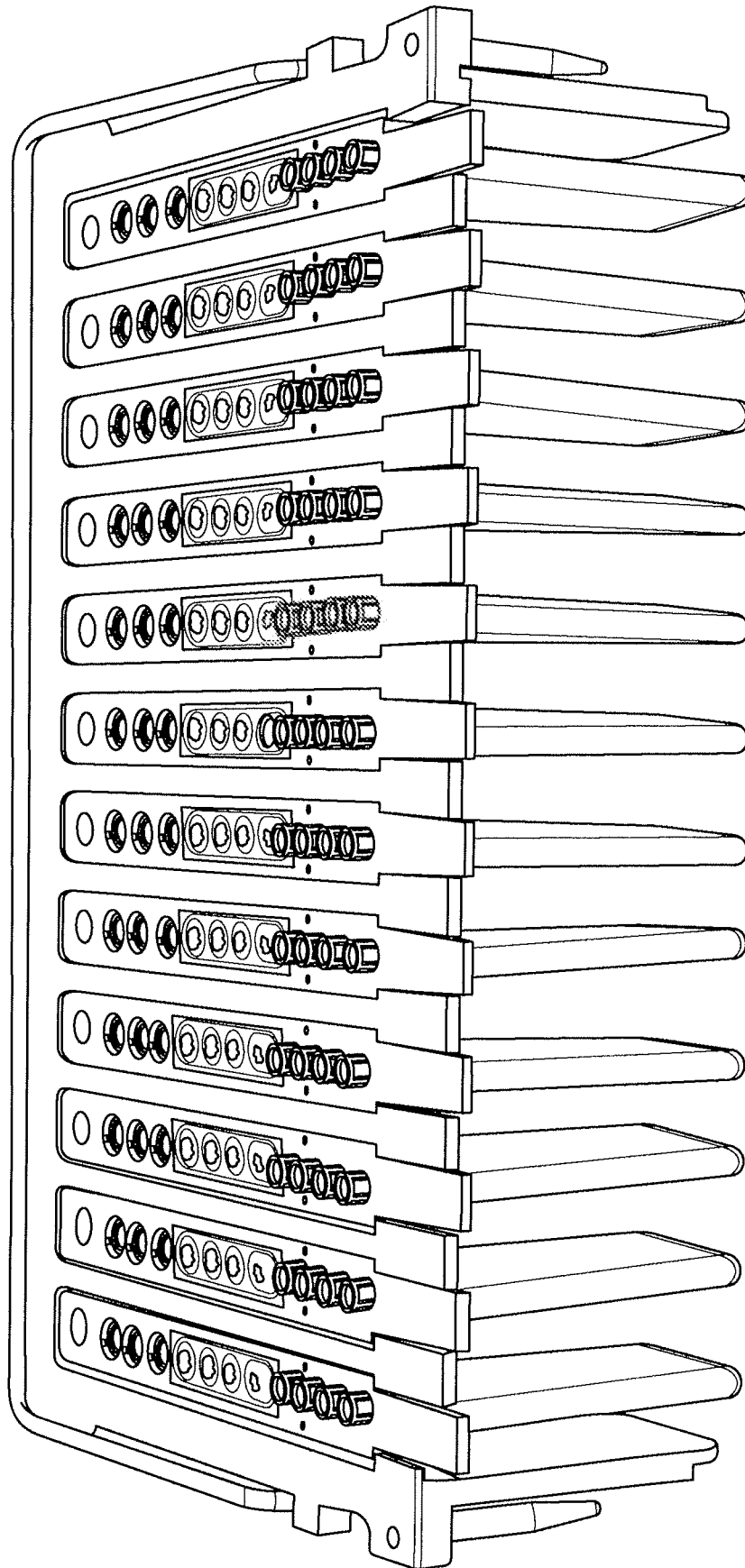


FIG. 8D

U.S. Patent

Apr. 28, 2020

Sheet 14 of 121

US 10,632,466 B1

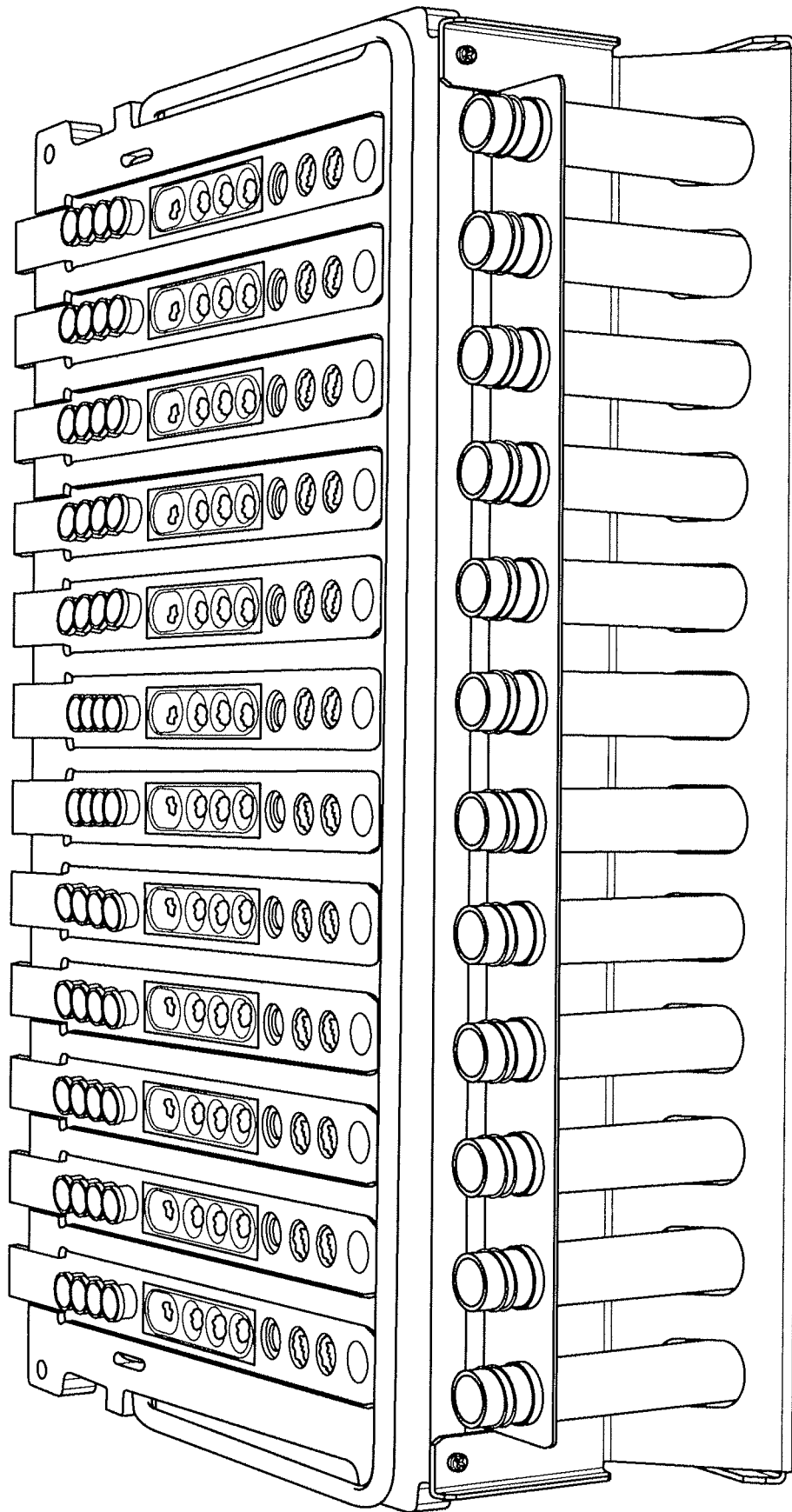


FIG. 8E

U.S. Patent

Apr. 28, 2020

Sheet 15 of 121

US 10,632,466 B1

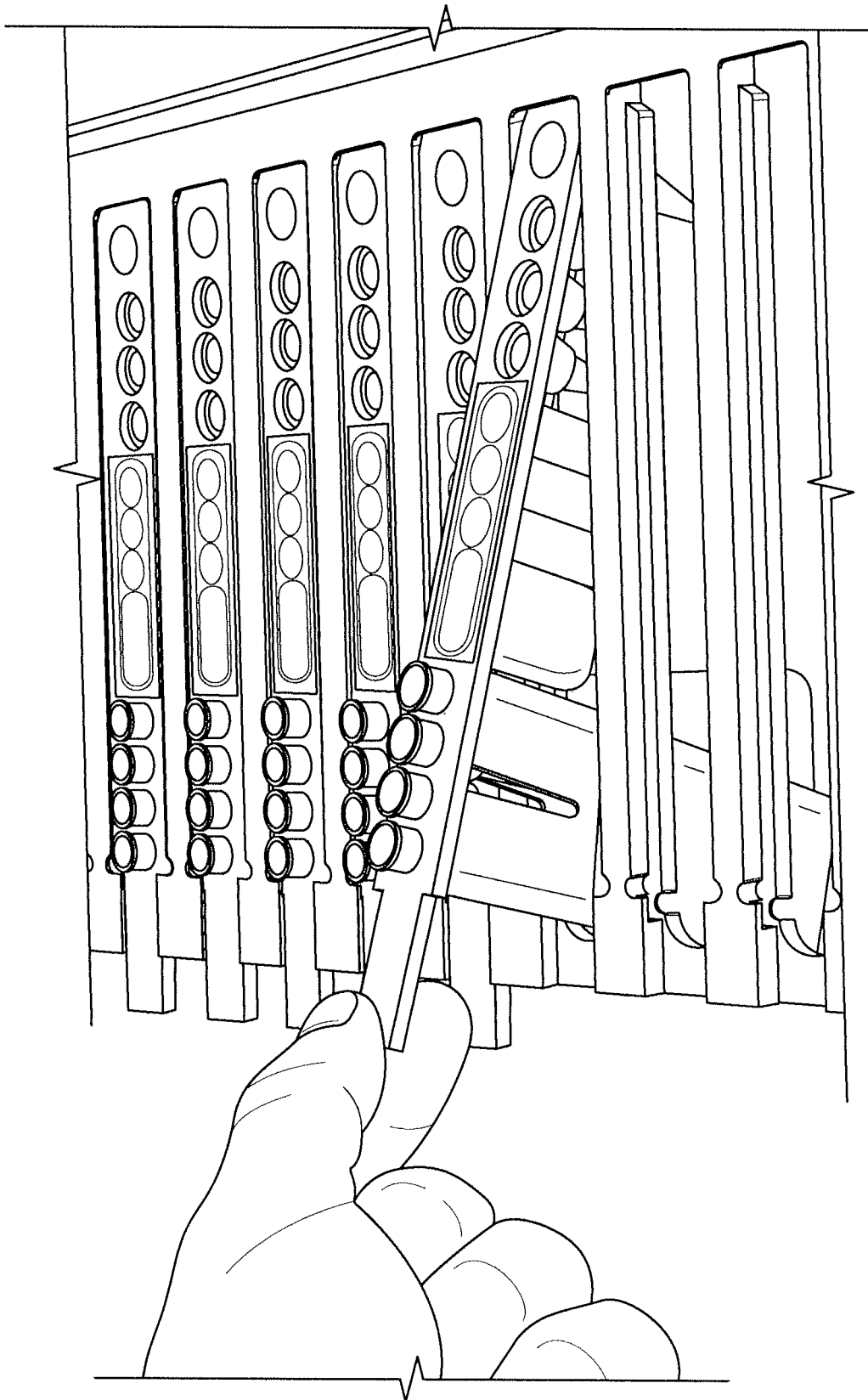


FIG. 8F

U.S. Patent

Apr. 28, 2020

Sheet 16 of 121

US 10,632,466 B1

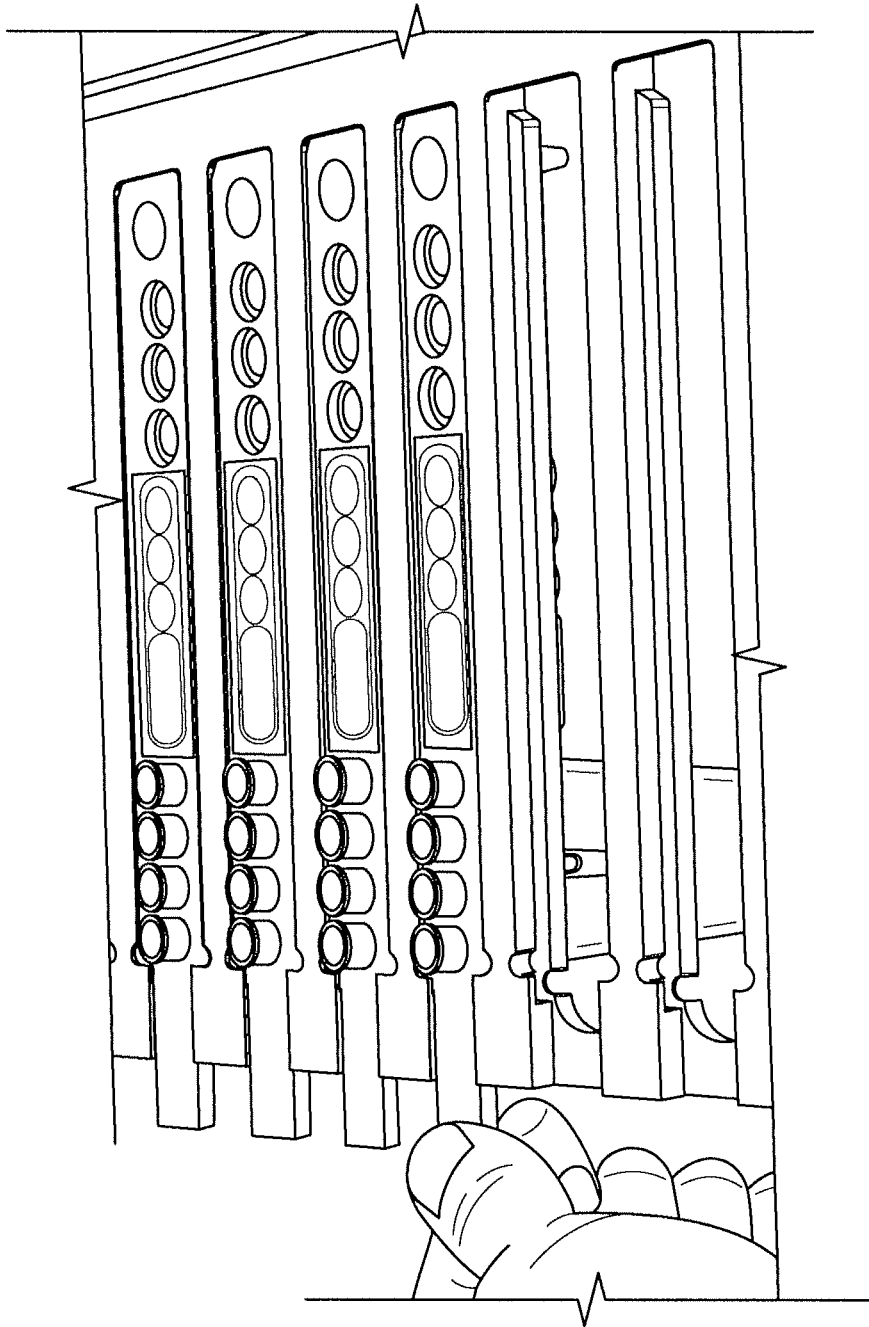


FIG. 8G

U.S. Patent

Apr. 28, 2020

Sheet 17 of 121

US 10,632,466 B1

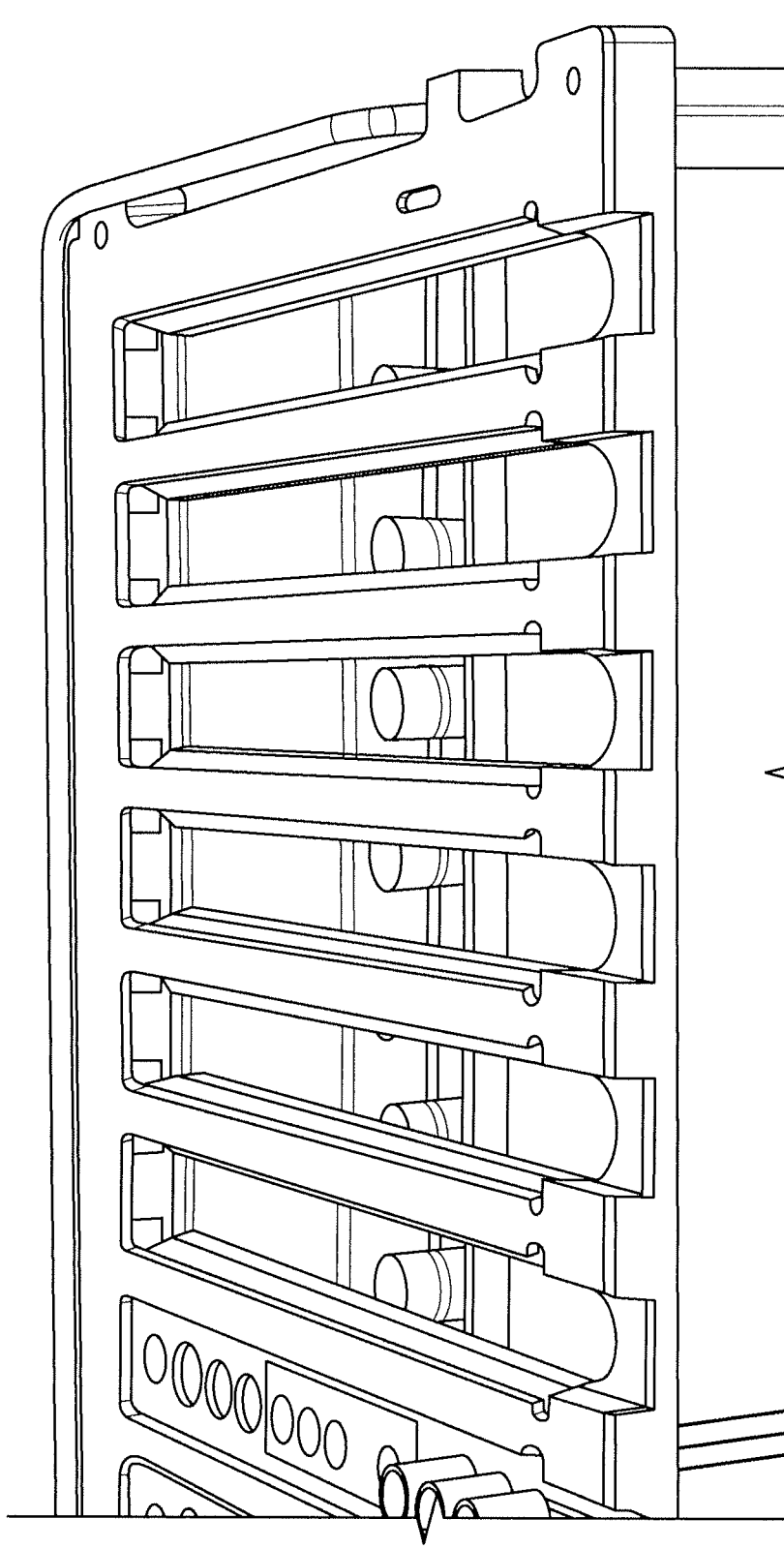


FIG. 8H

U.S. Patent

Apr. 28, 2020

Sheet 18 of 121

US 10,632,466 B1

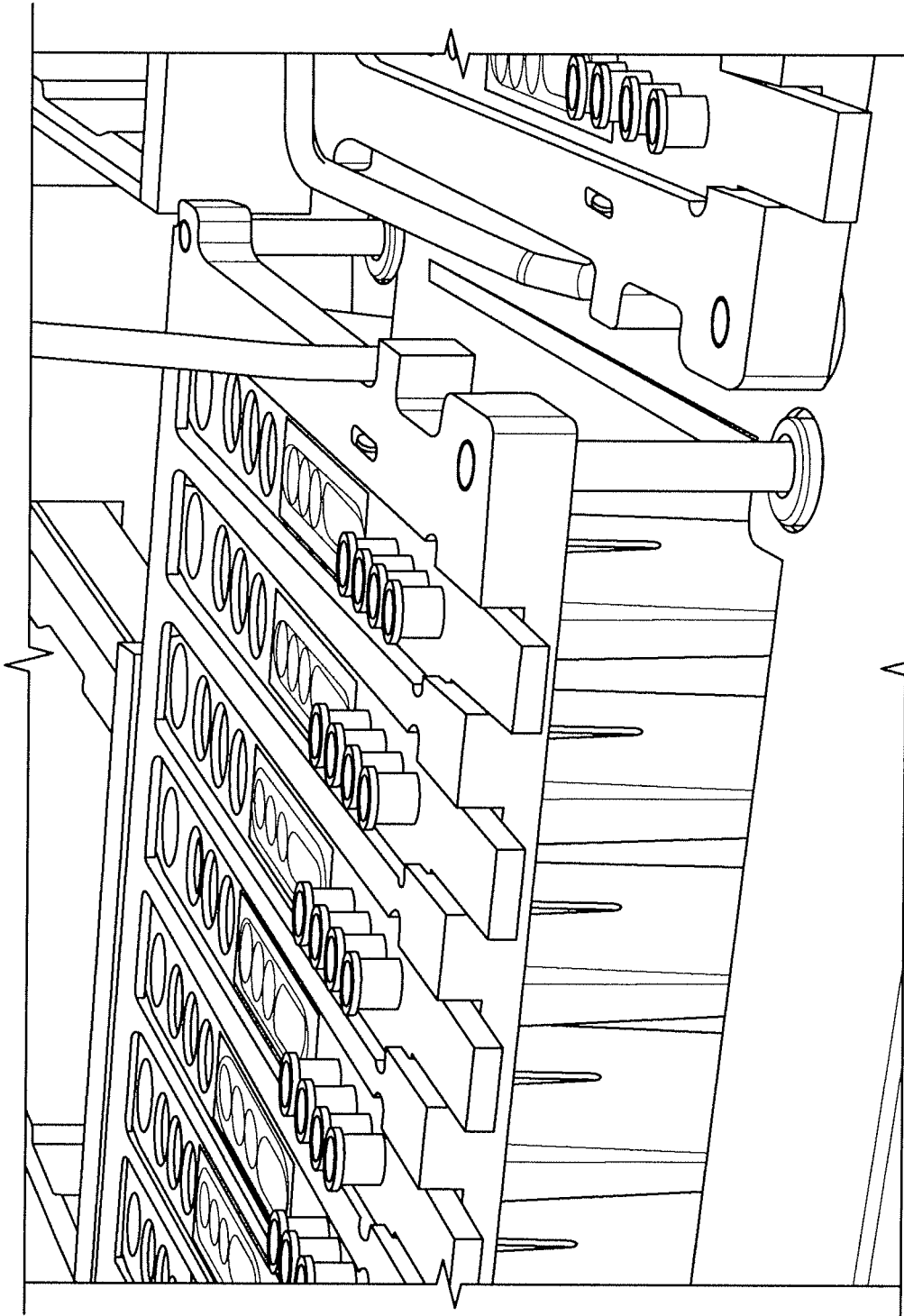


FIG. 8I

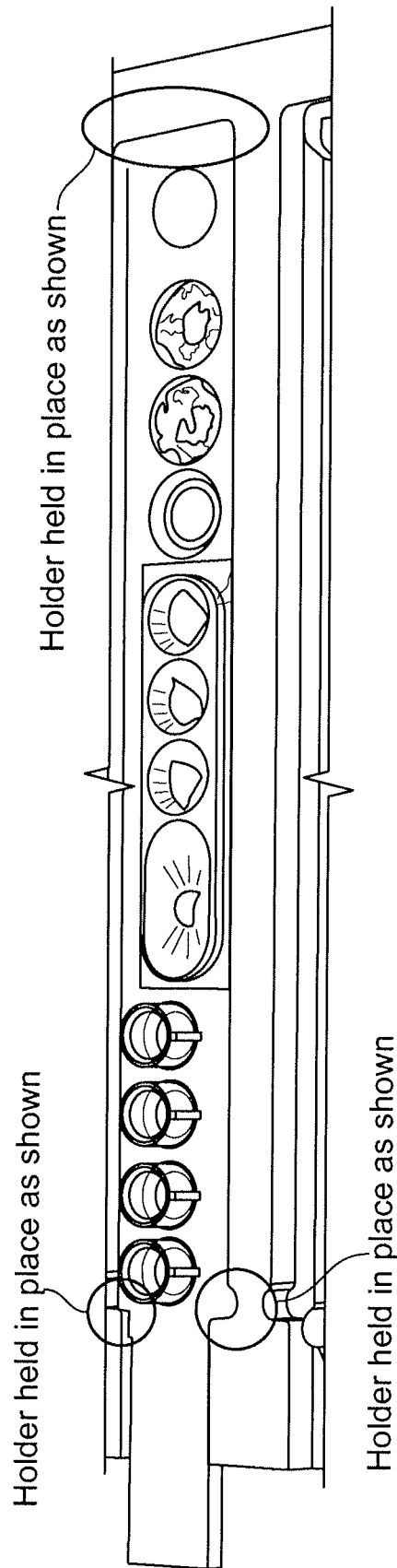


FIG. 8J

U.S. Patent

Apr. 28, 2020

Sheet 20 of 121

US 10,632,466 B1

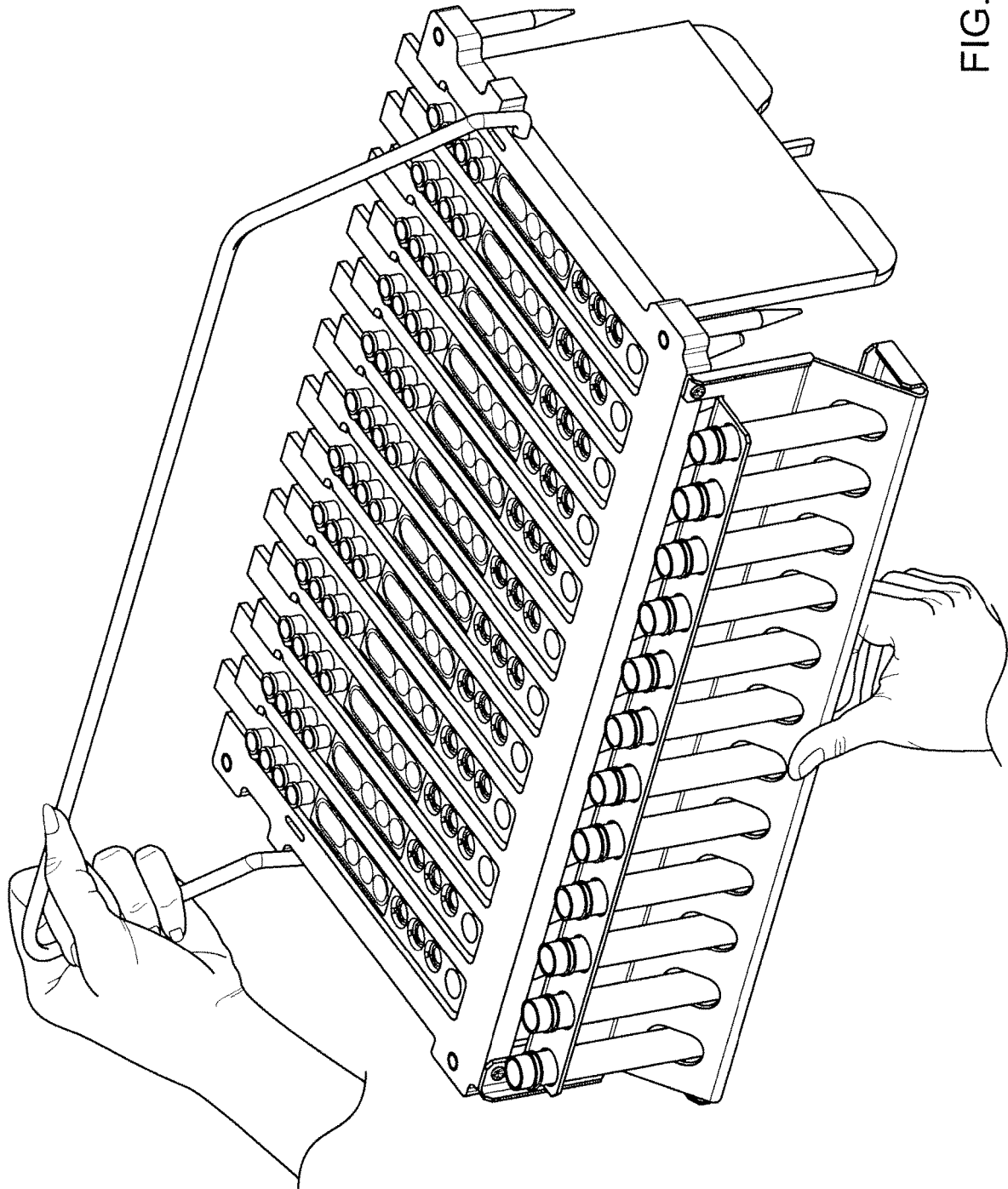


FIG. 8K

U.S. Patent

Apr. 28, 2020

Sheet 21 of 121

US 10,632,466 B1

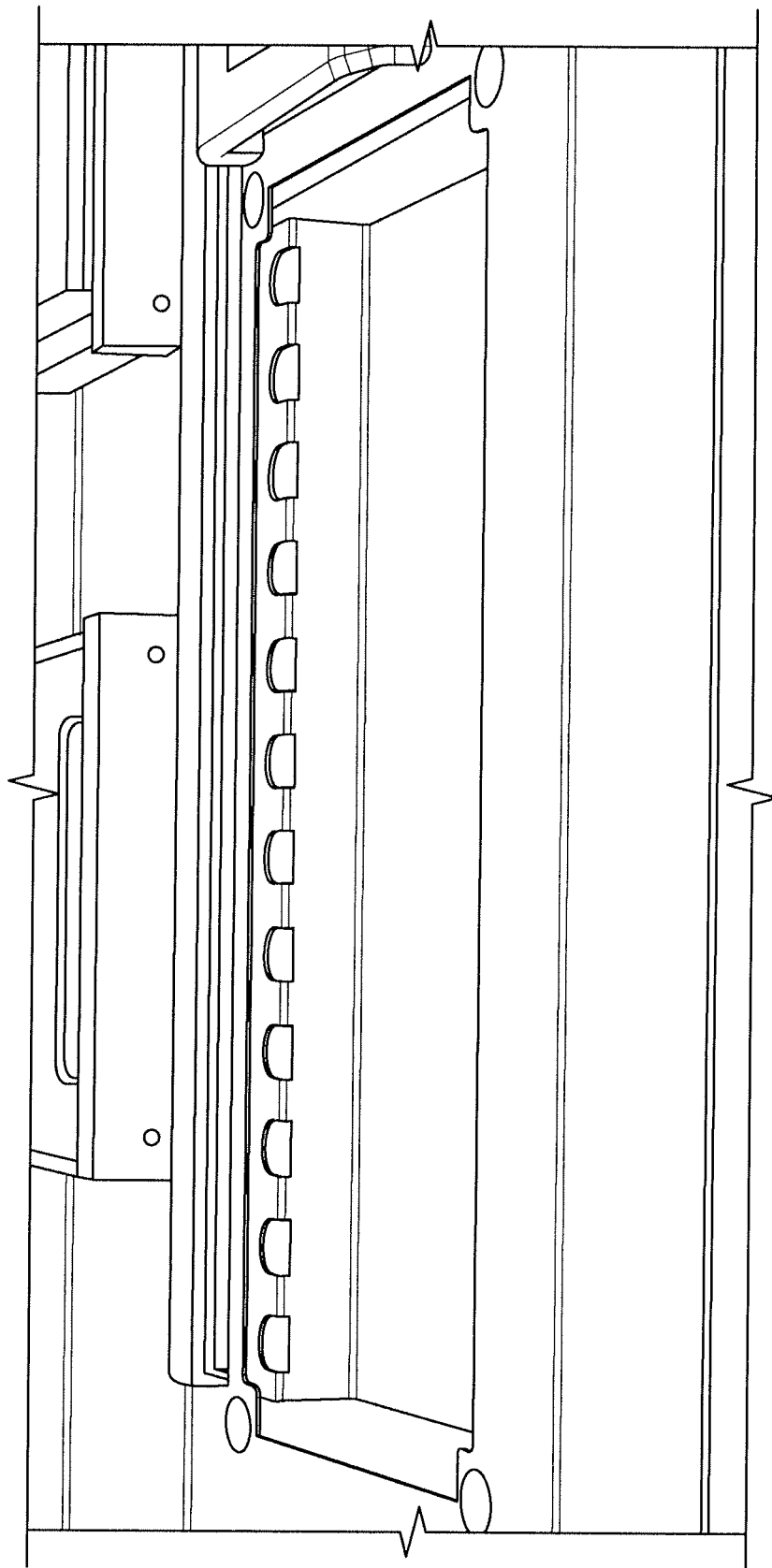


FIG. 9

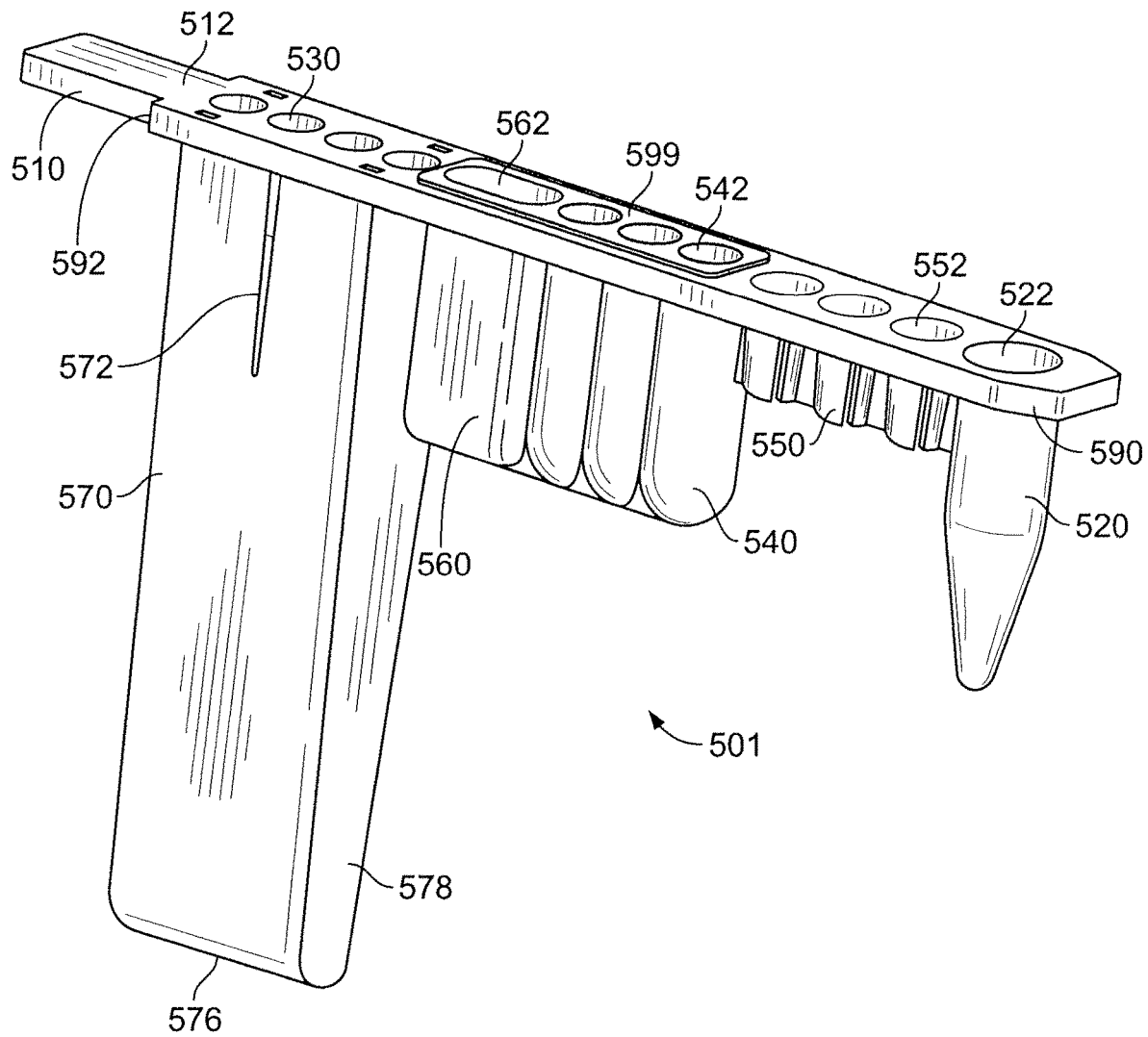


FIG. 10A

U.S. Patent

Apr. 28, 2020

Sheet 23 of 121

US 10,632,466 B1

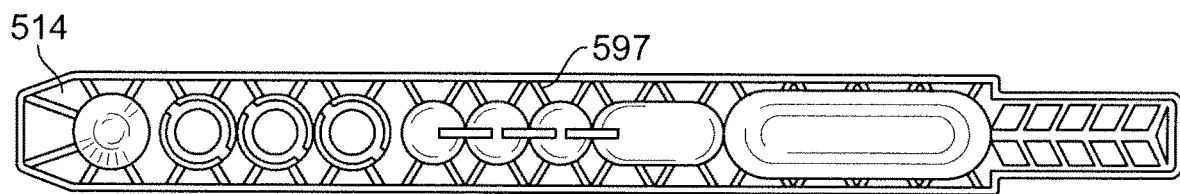


FIG. 10B

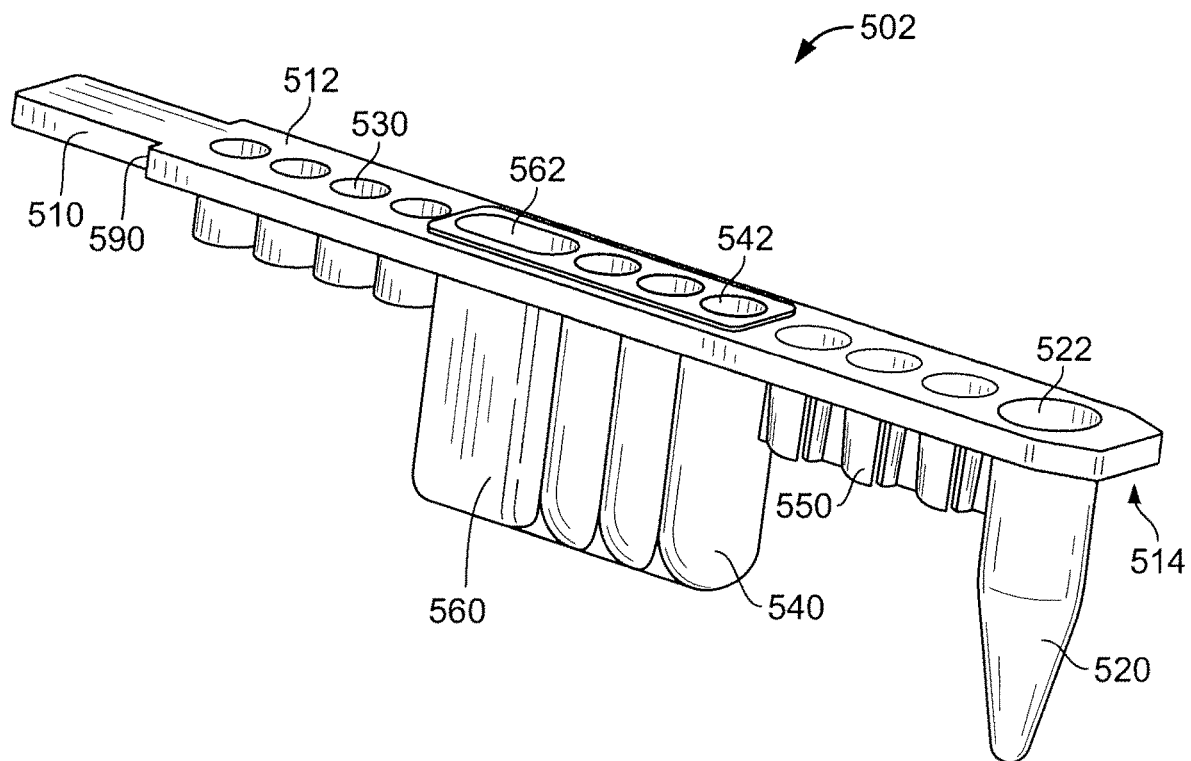


FIG. 11

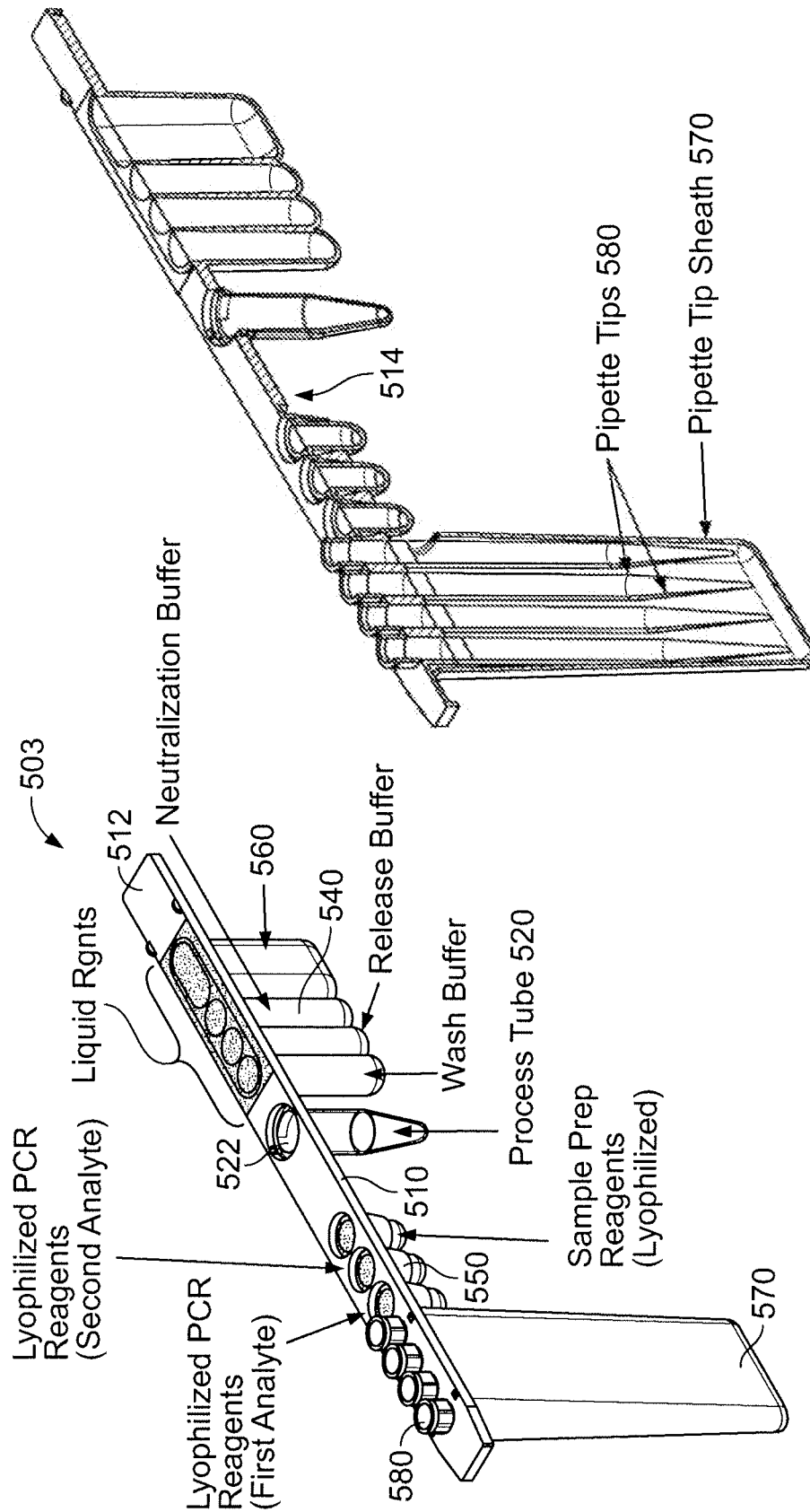


FIG. 12B

FIG. 12A

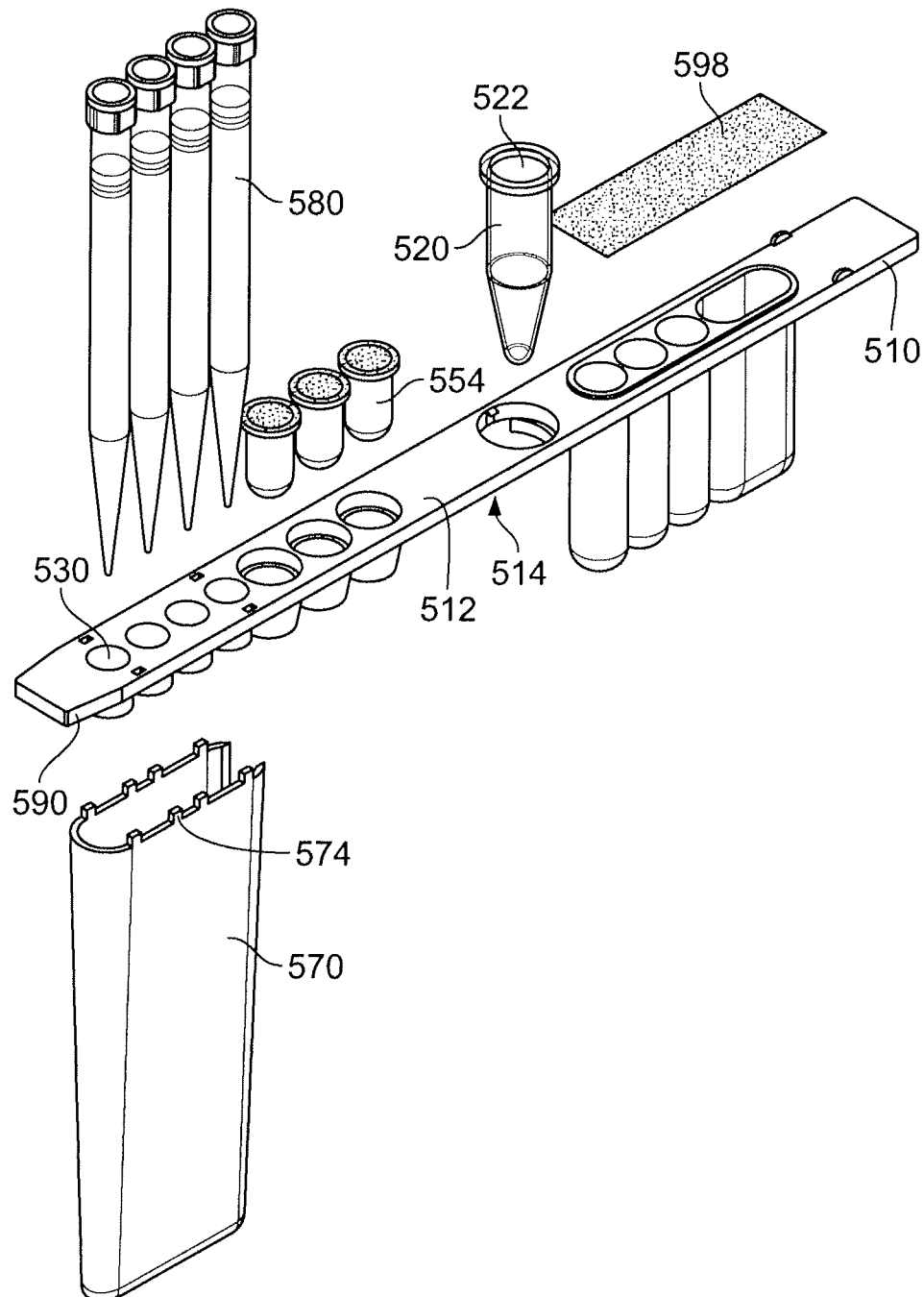


FIG. 12C

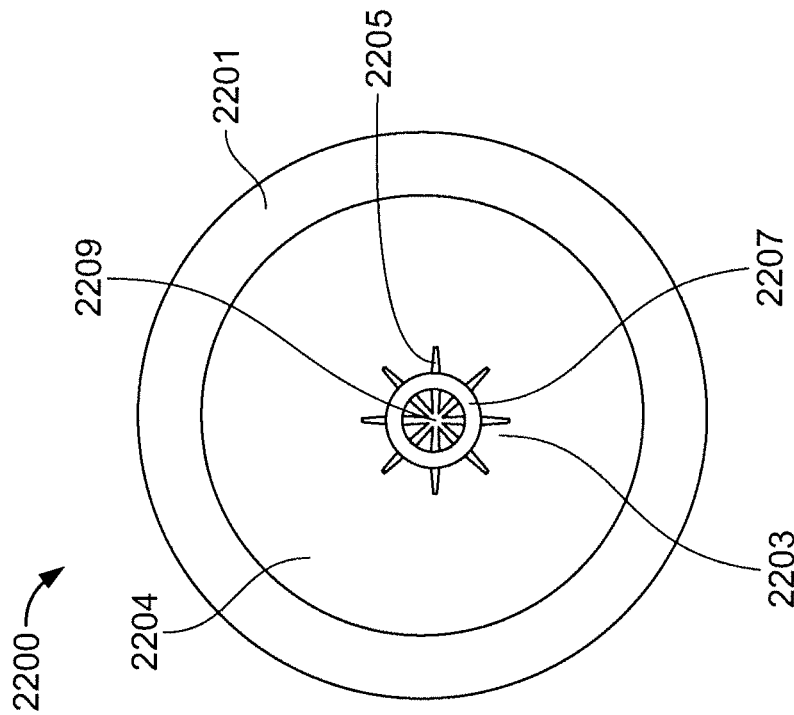


FIG. 13B

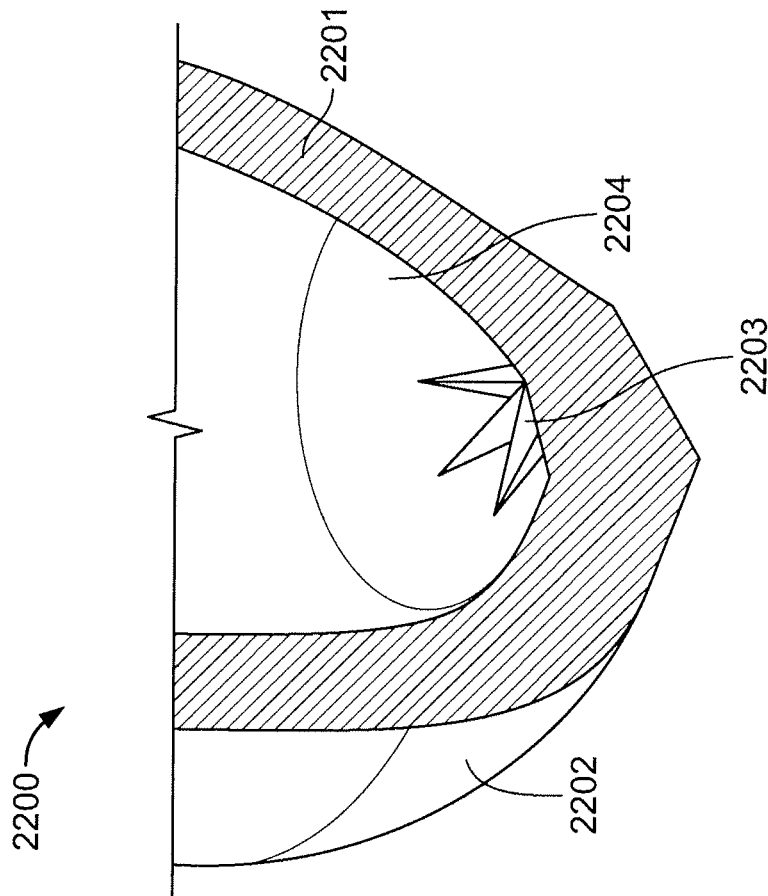


FIG. 13A

U.S. Patent

Apr. 28, 2020

Sheet 28 of 121

US 10,632,466 B1

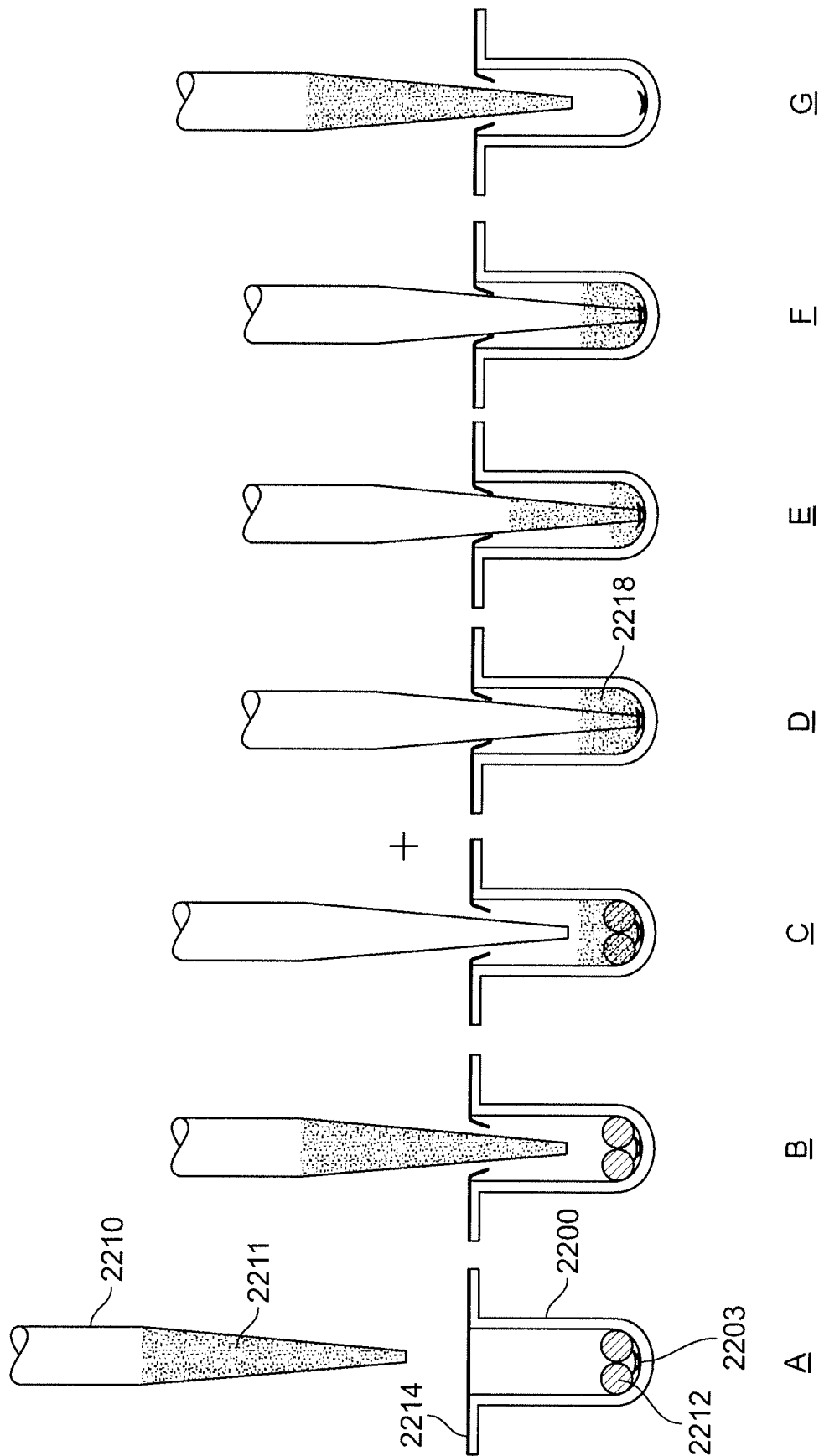


FIG. 14

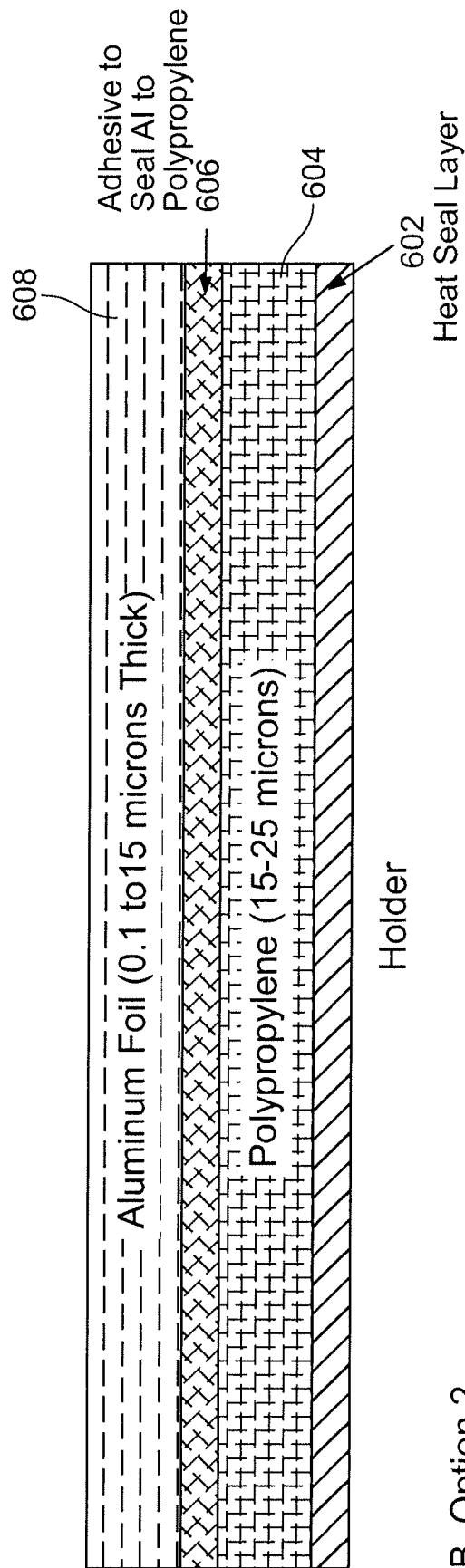
U.S. Patent

Apr. 28, 2020

Sheet 29 of 121

US 10,632,466 B1

A. Option 1



B. Option 2

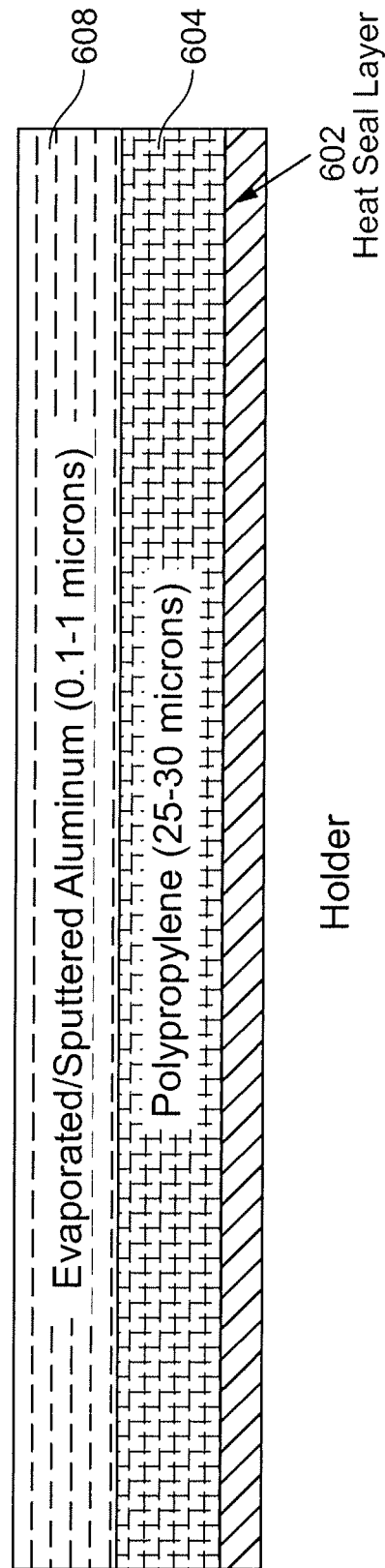


FIG. 15

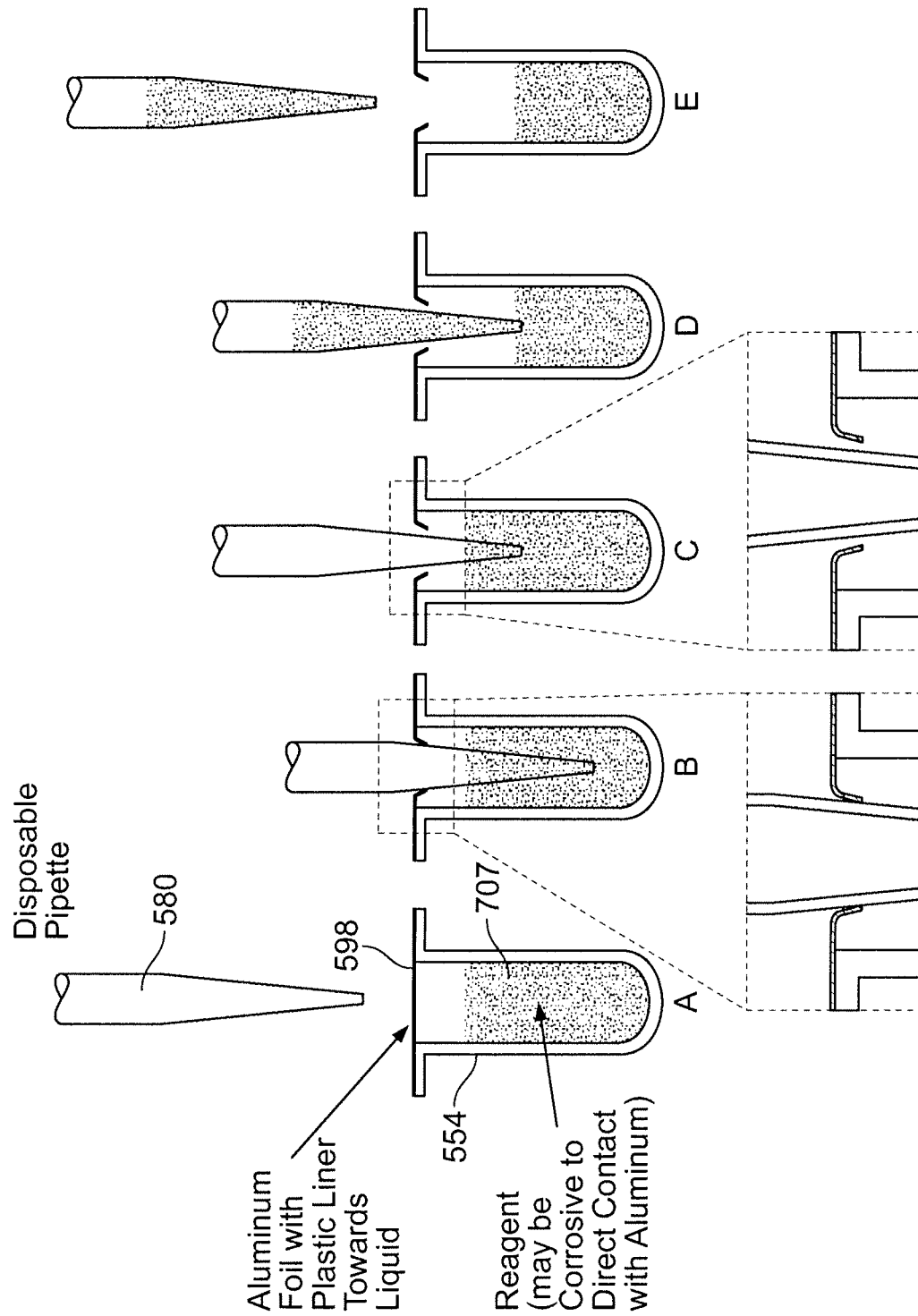


FIG. 16

U.S. Patent

Apr. 28, 2020

Sheet 31 of 121

US 10,632,466 B1

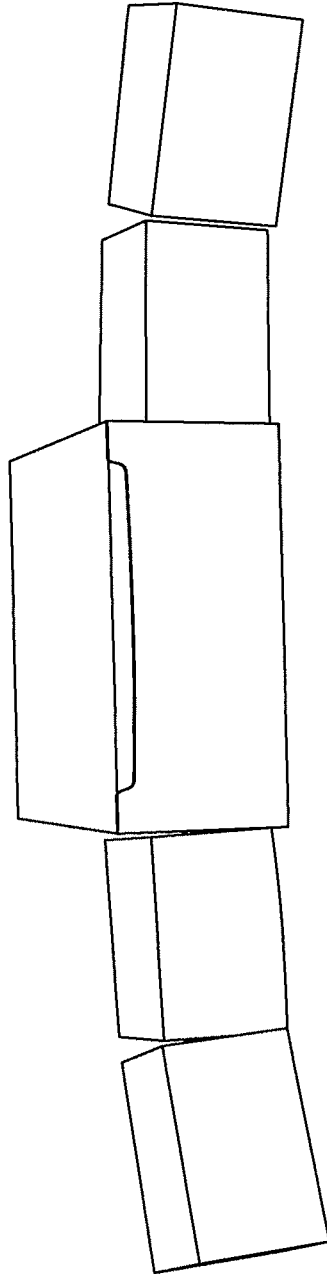


FIG. 17A

U.S. Patent

Apr. 28, 2020

Sheet 32 of 121

US 10,632,466 B1

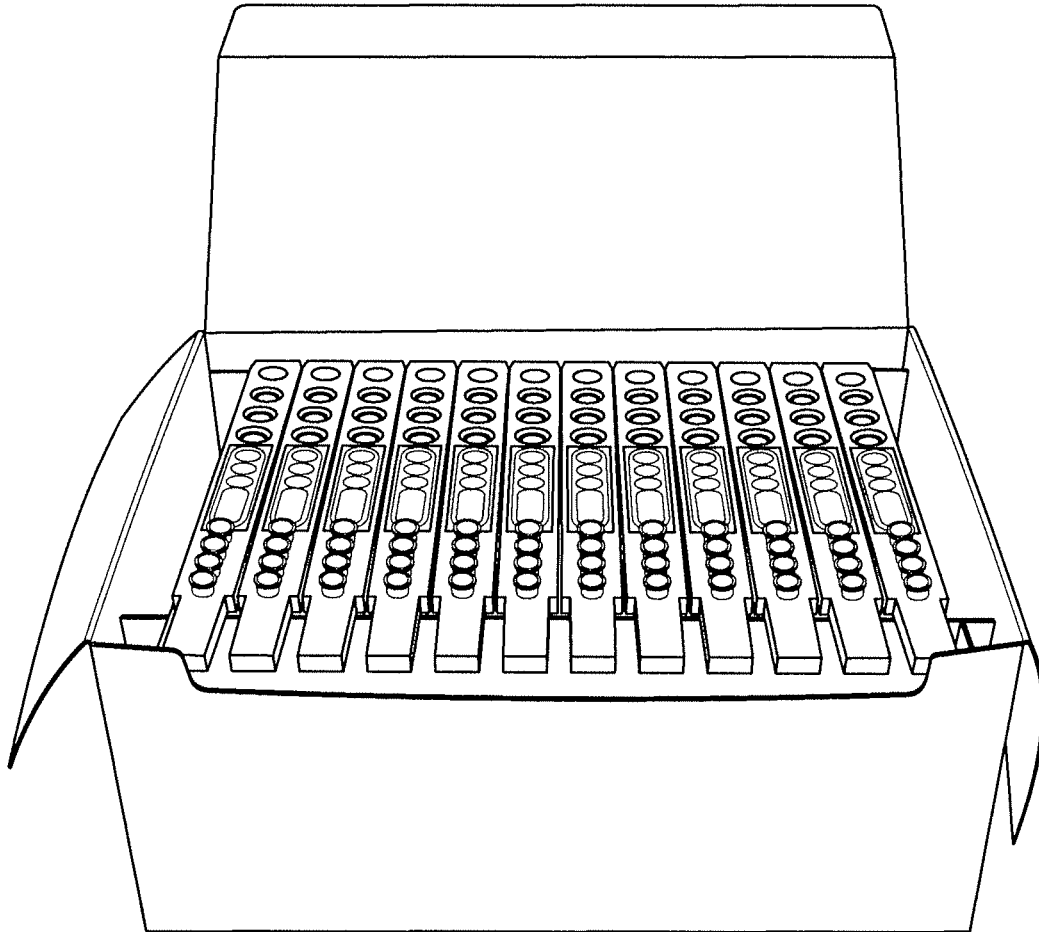


FIG. 17B

U.S. Patent

Apr. 28, 2020

Sheet 33 of 121

US 10,632,466 B1

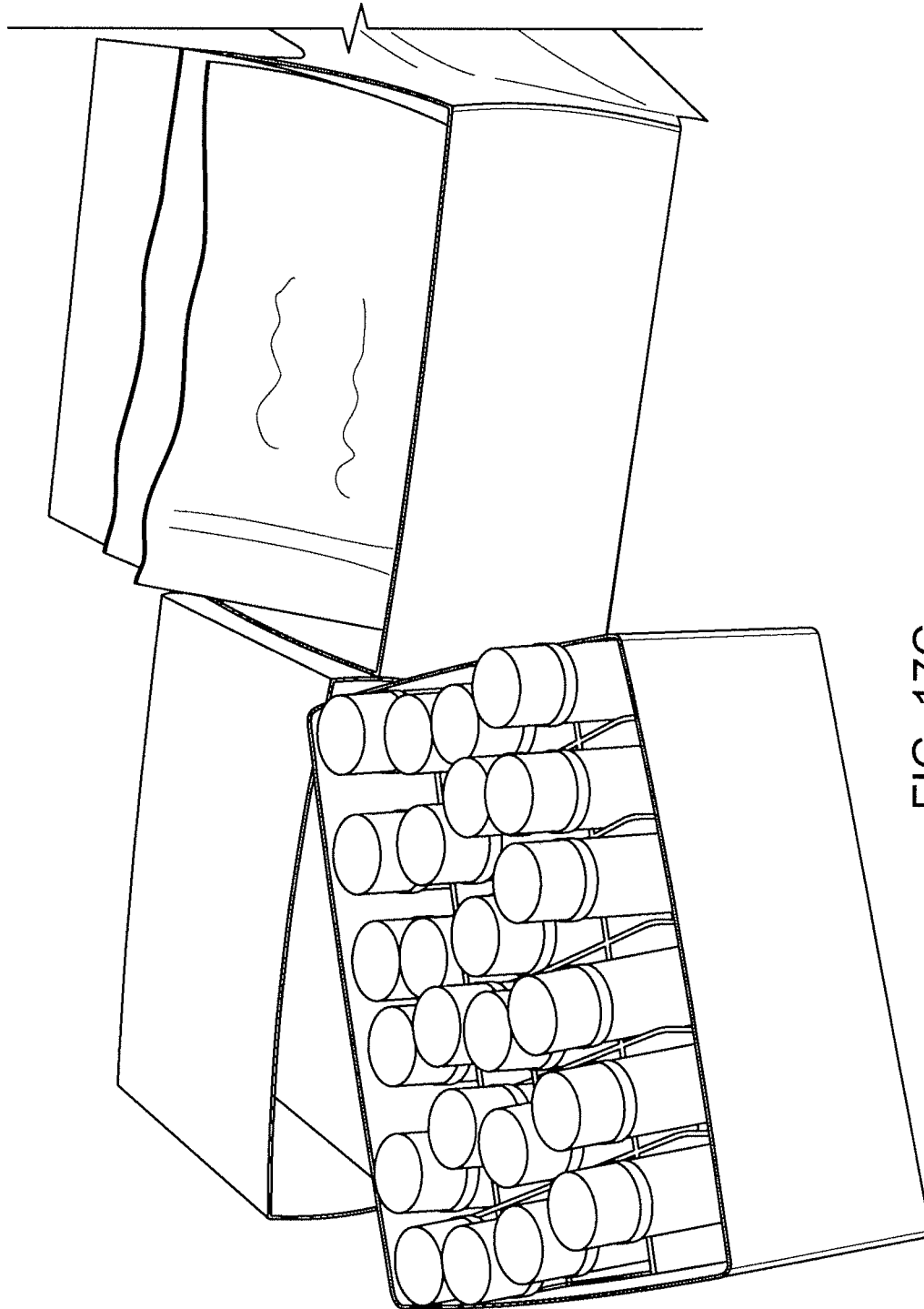


FIG. 17C

U.S. Patent

Apr. 28, 2020

Sheet 34 of 121

US 10,632,466 B1

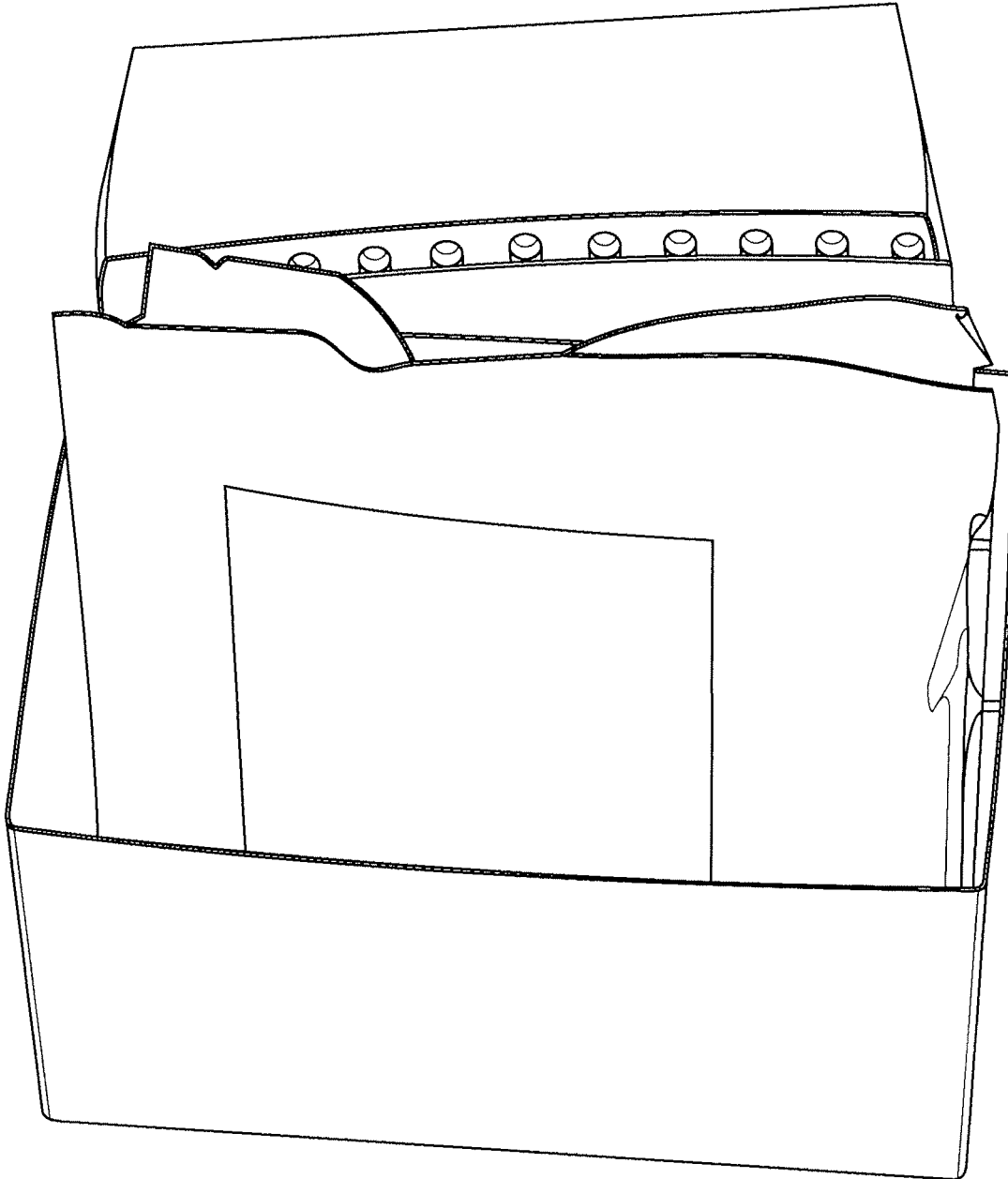
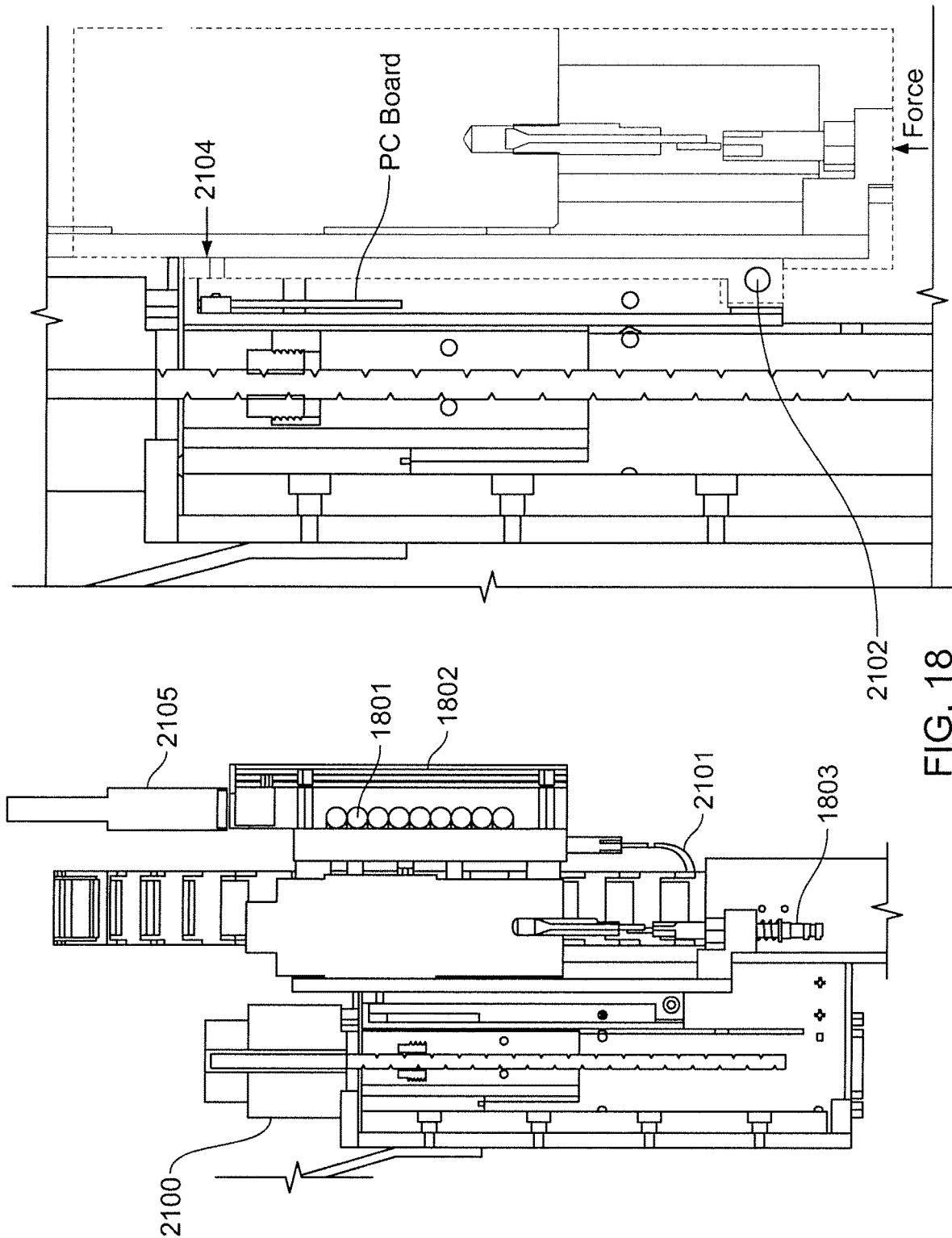


FIG. 17D



U.S. Patent

Apr. 28, 2020

Sheet 36 of 121

US 10,632,466 B1

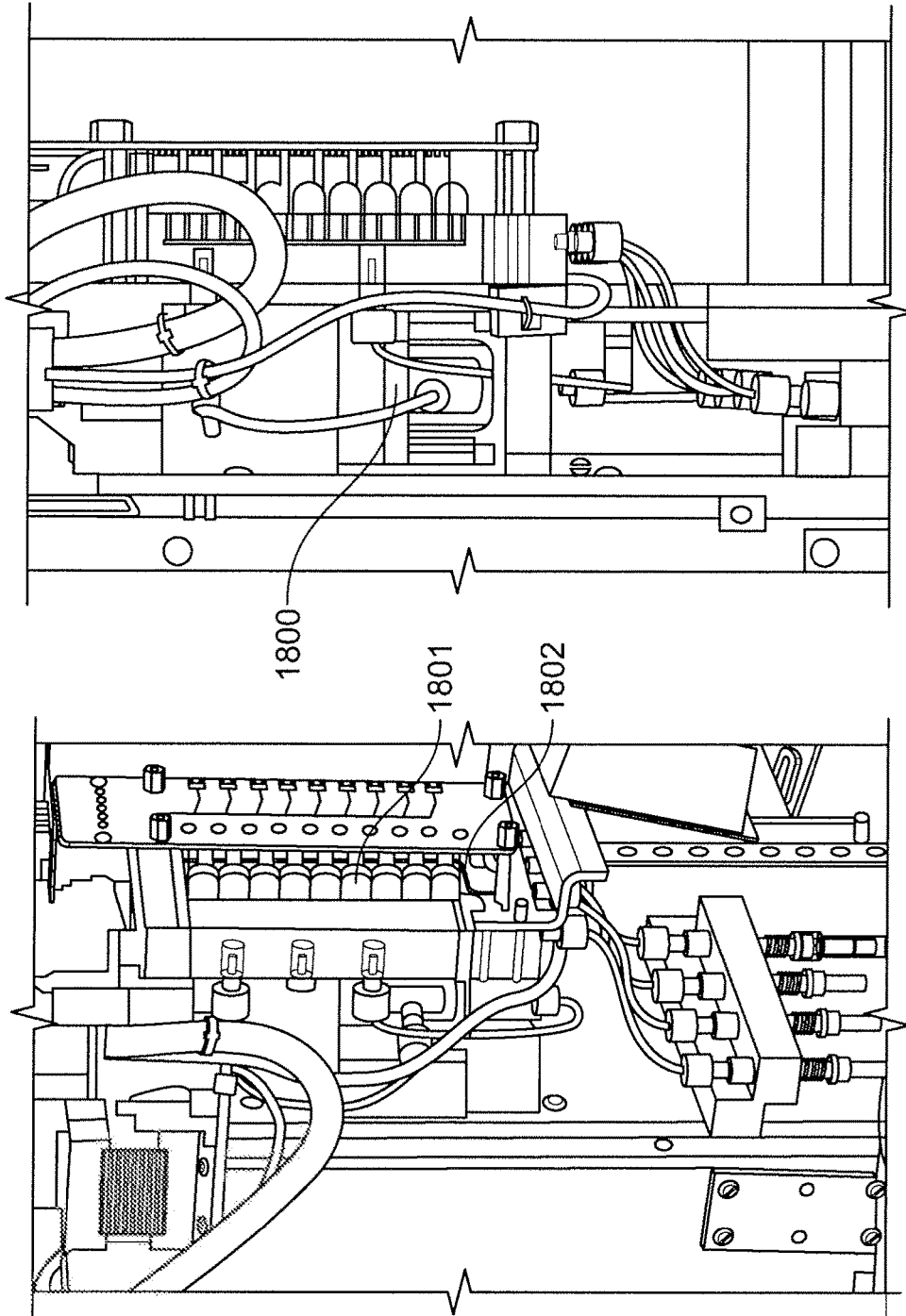
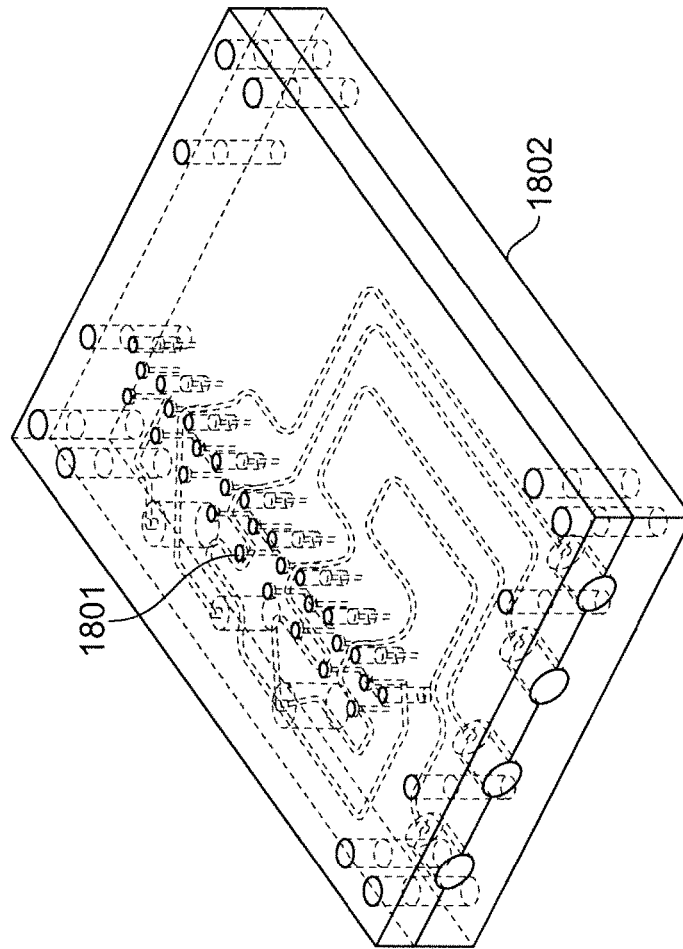
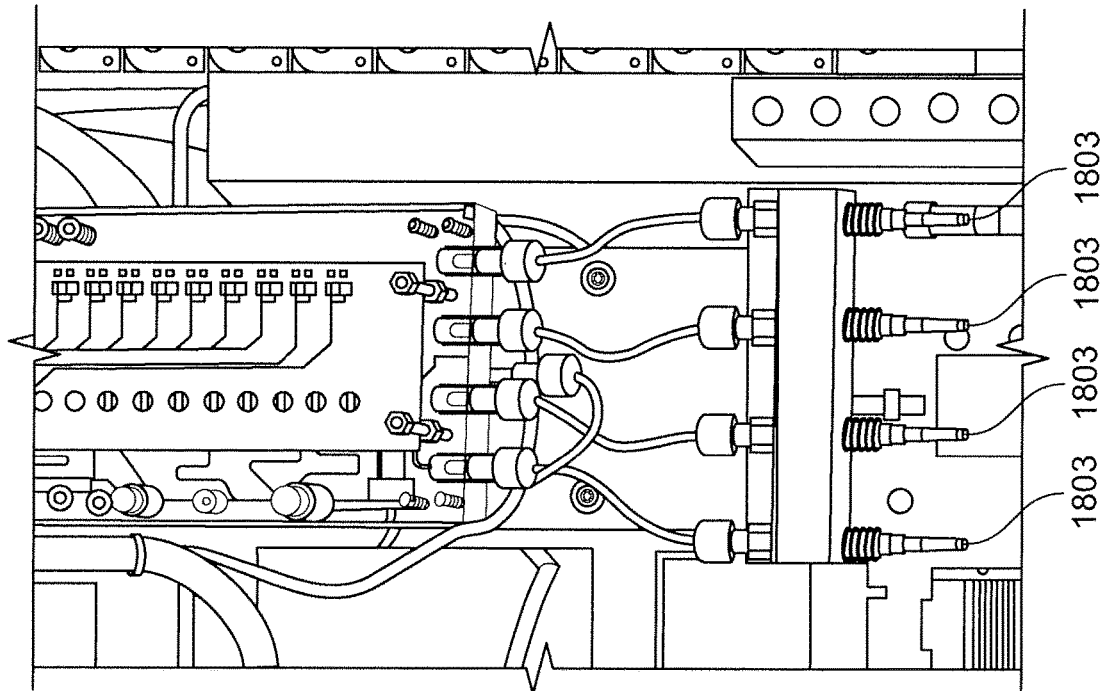


FIG. 19B

FIG. 19A

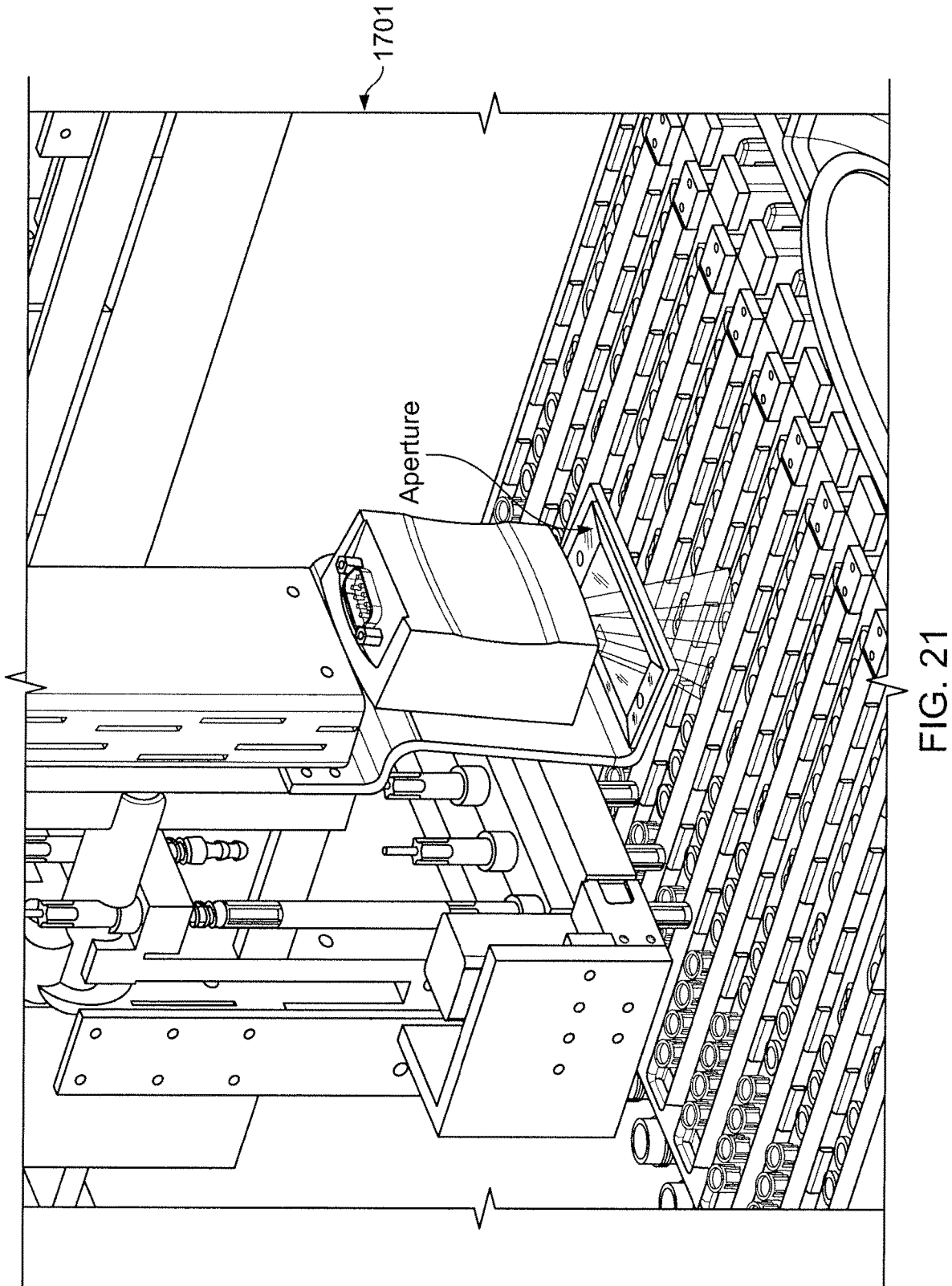


U.S. Patent

Apr. 28, 2020

Sheet 38 of 121

US 10,632,466 B1



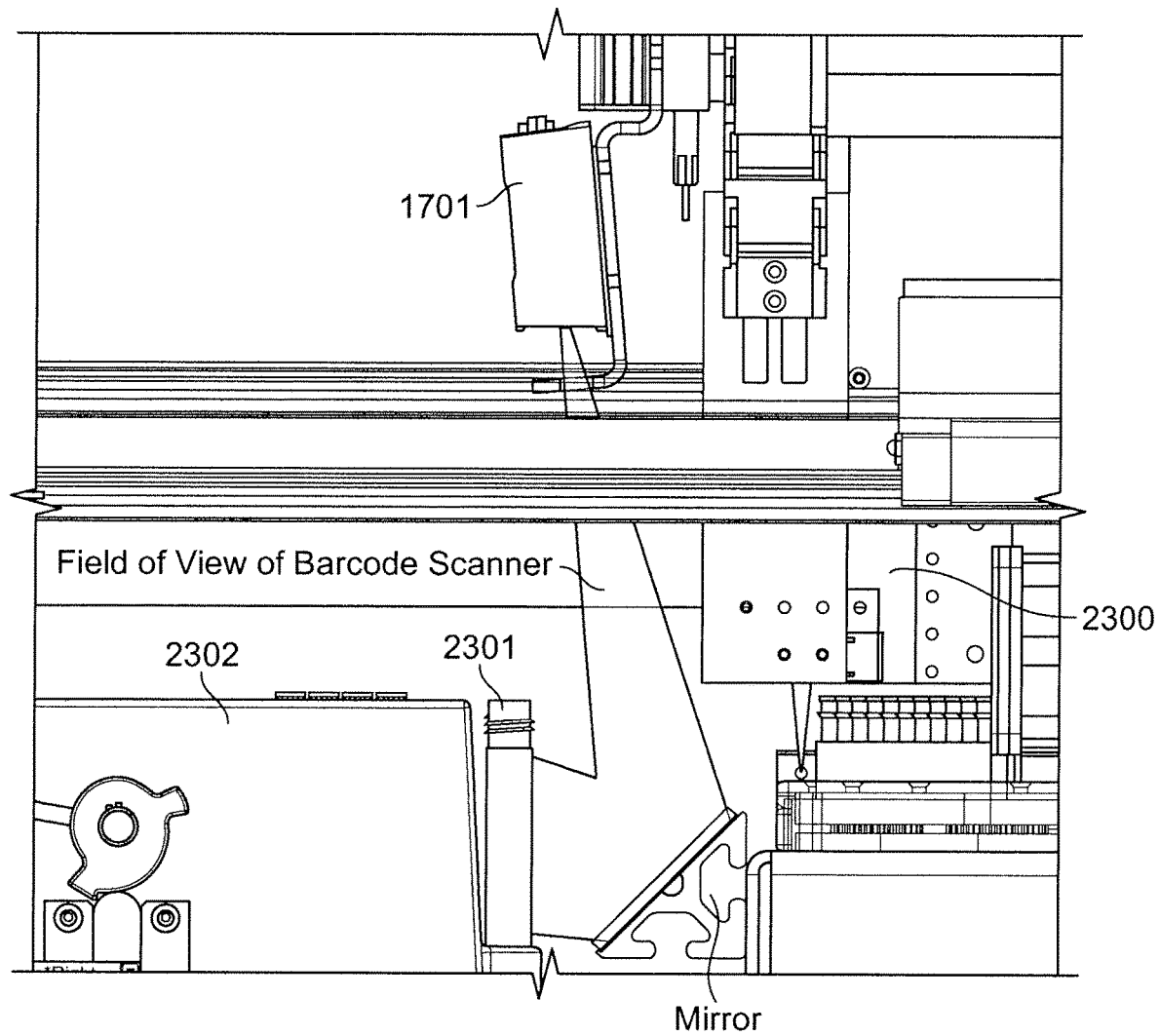


FIG. 22

U.S. Patent

Apr. 28, 2020

Sheet 40 of 121

US 10,632,466 B1

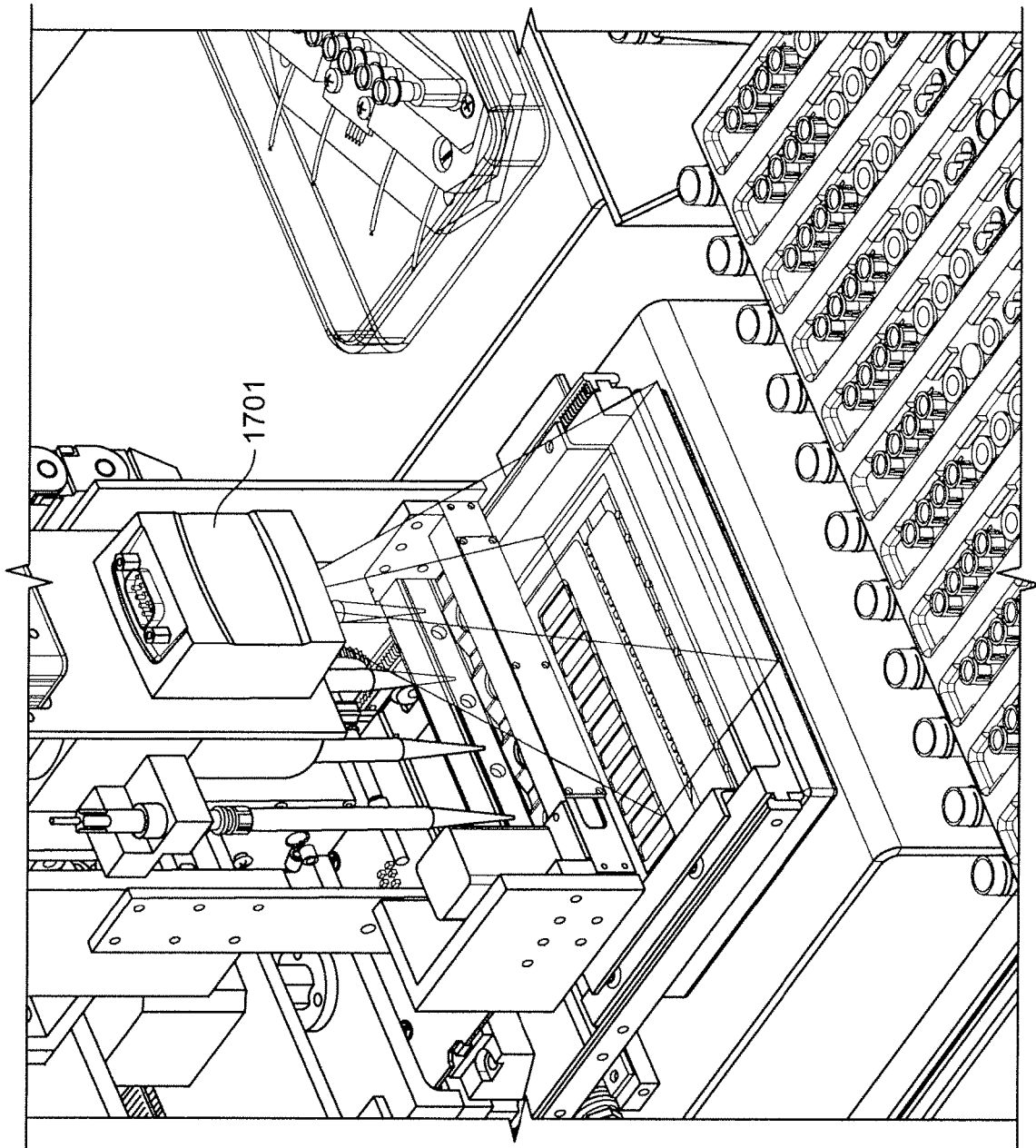


FIG. 23

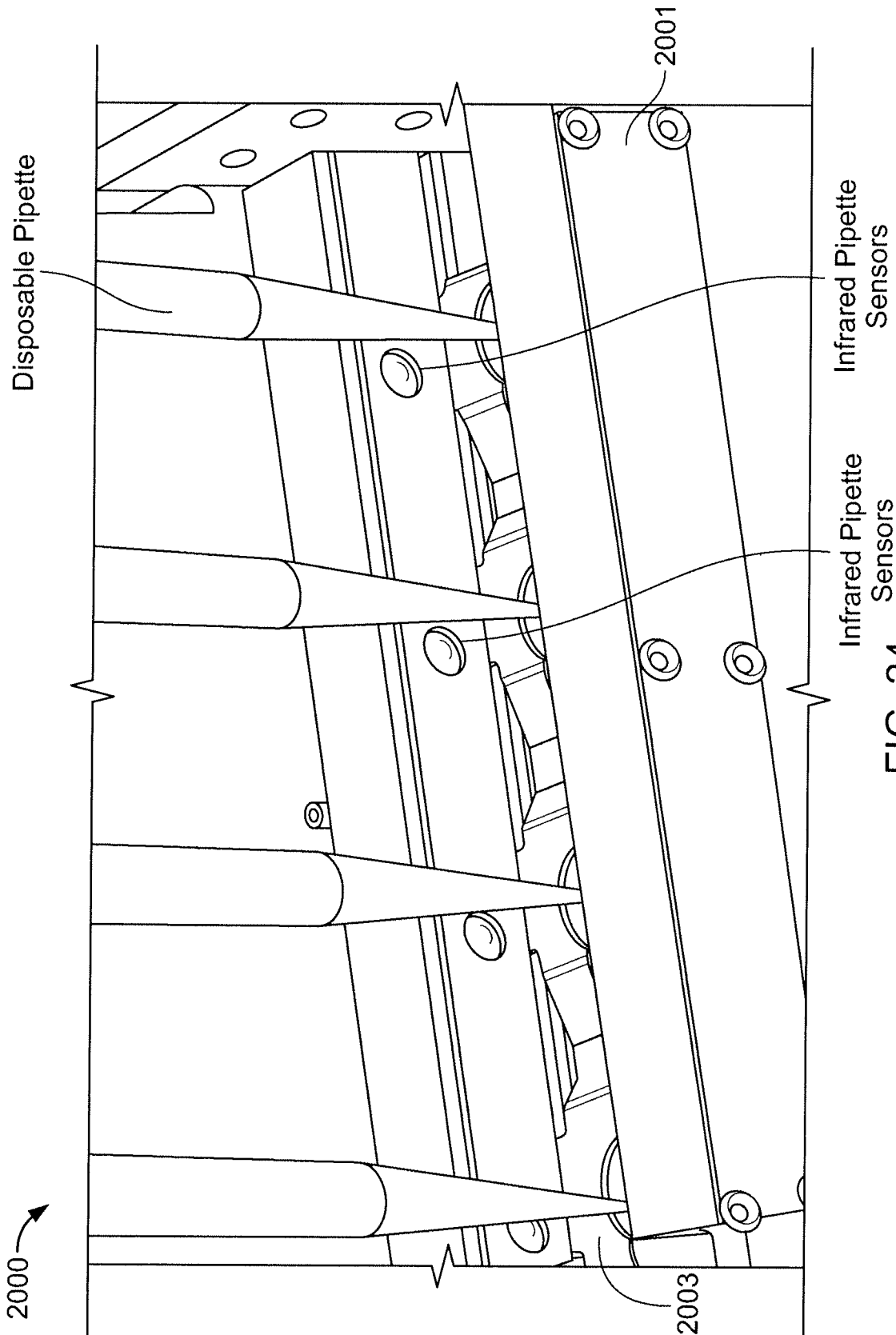


FIG. 24

U.S. Patent

Apr. 28, 2020

Sheet 42 of 121

US 10,632,466 B1

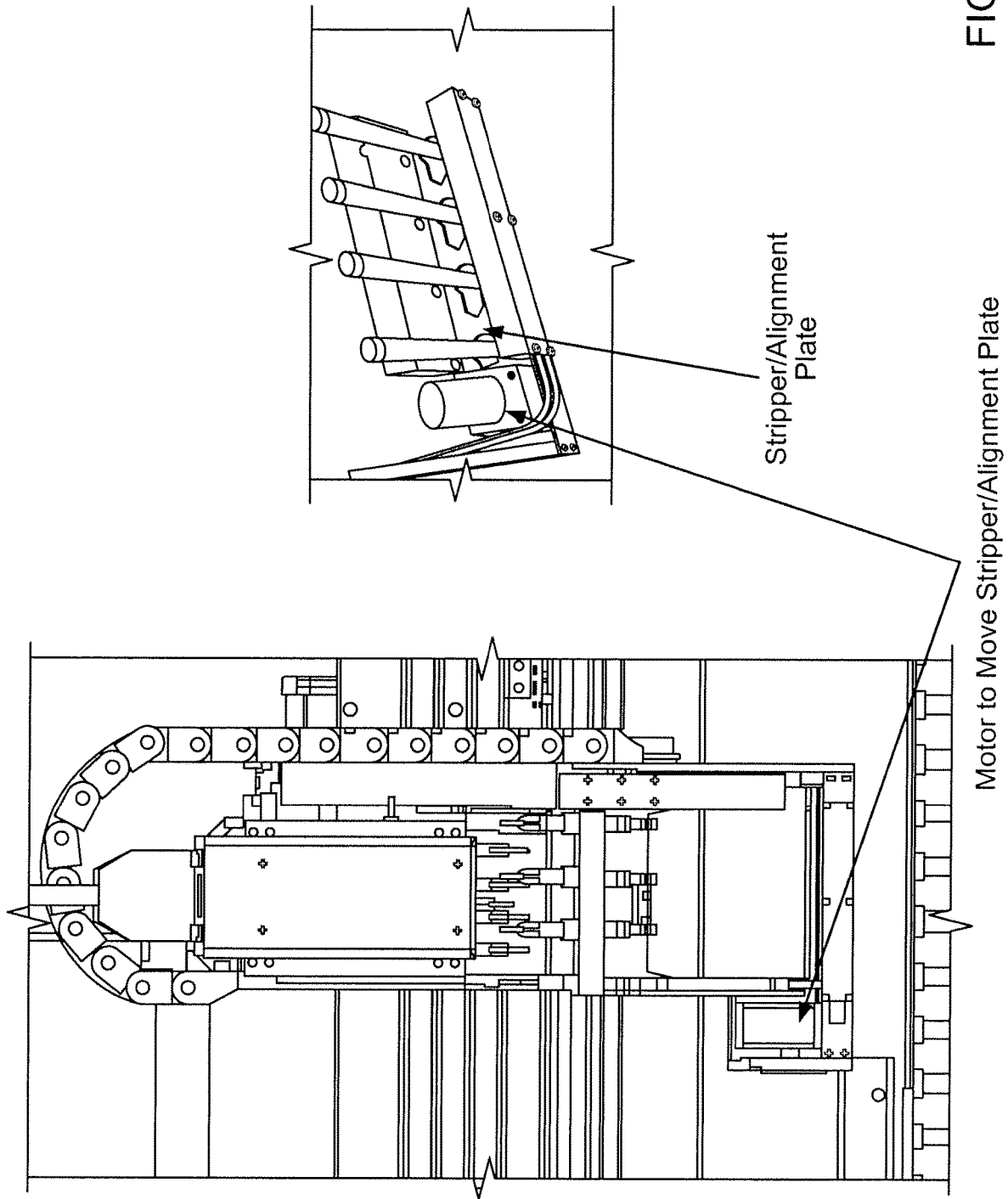


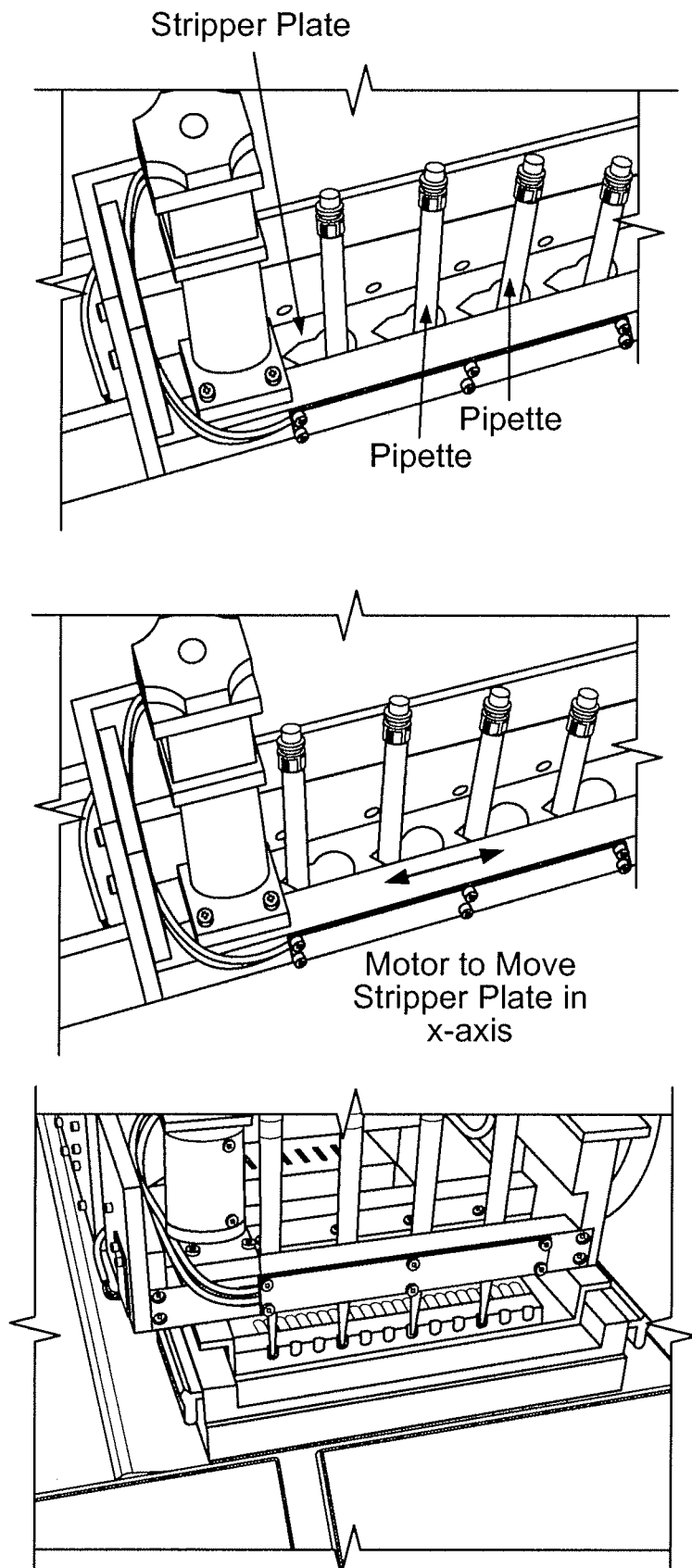
FIG. 25A

U.S. Patent

Apr. 28, 2020

Sheet 43 of 121

US 10,632,466 B1



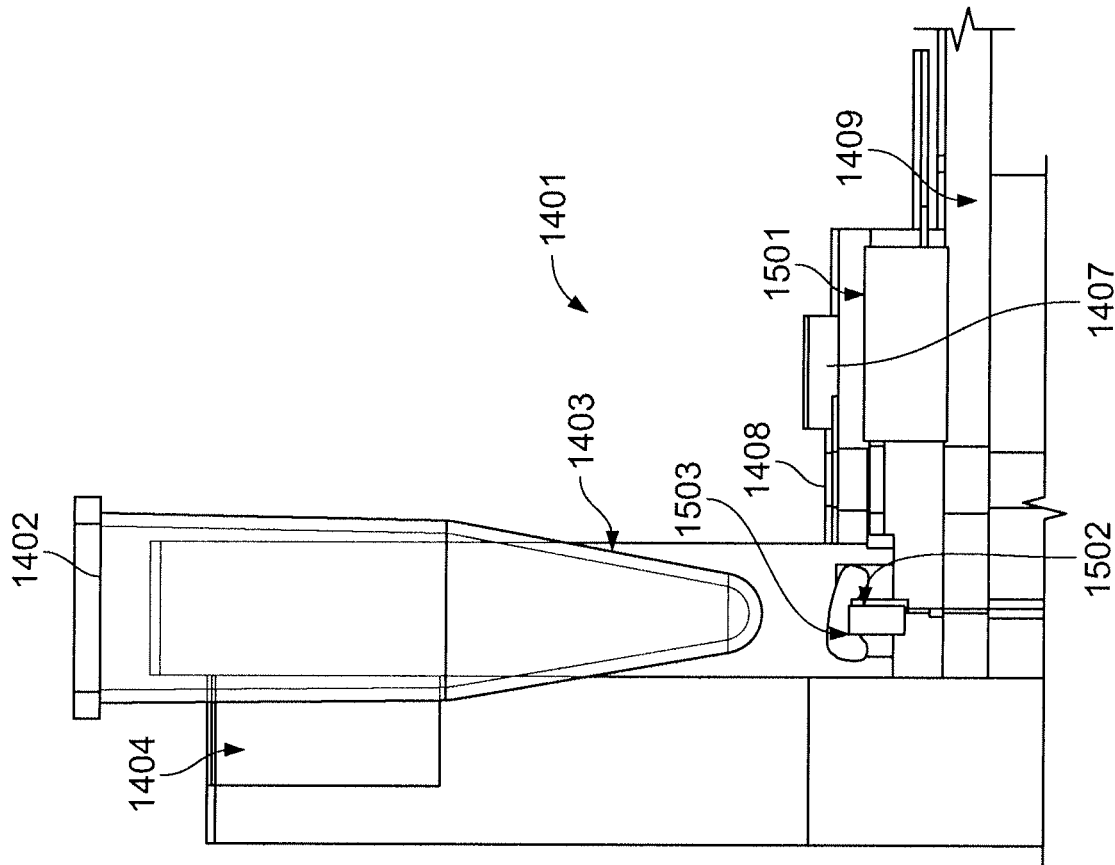
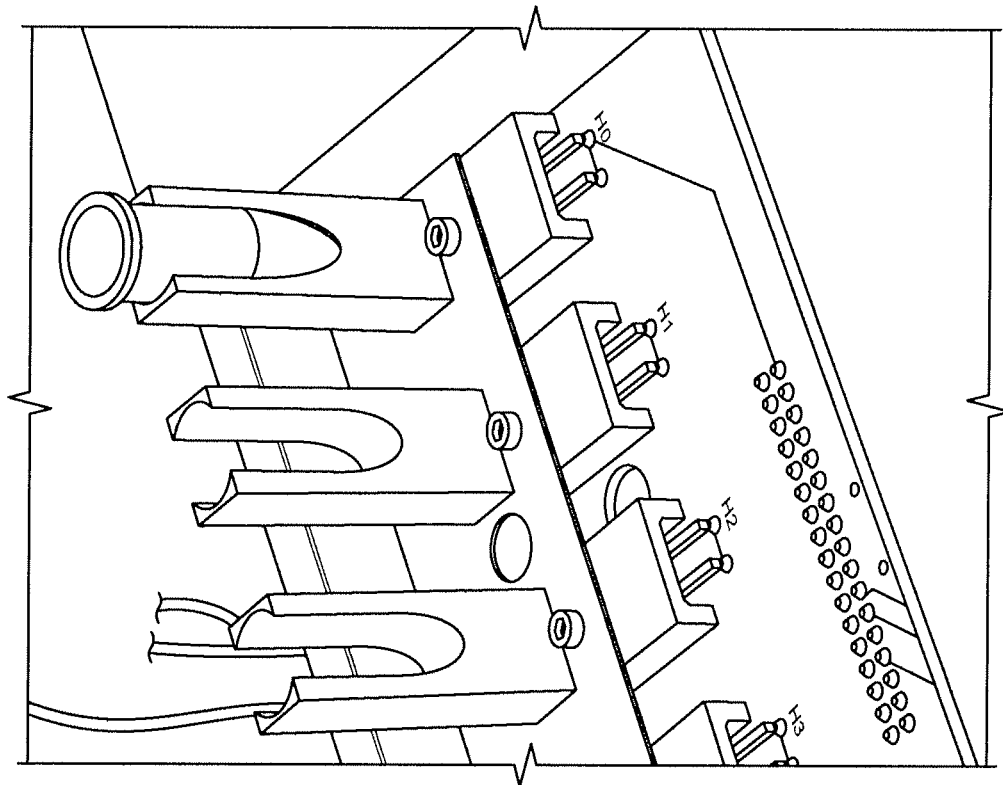


FIG. 26



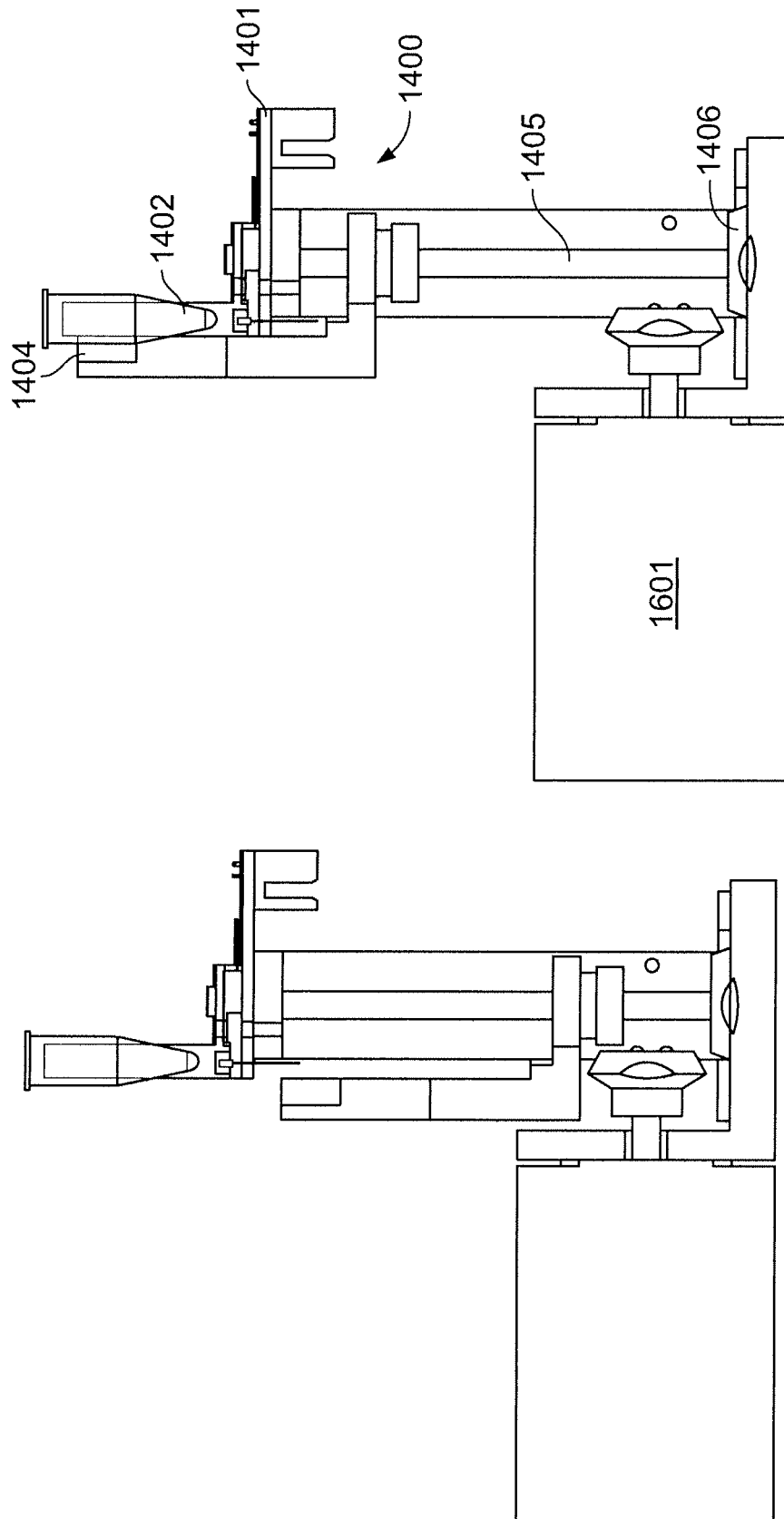


FIG. 27

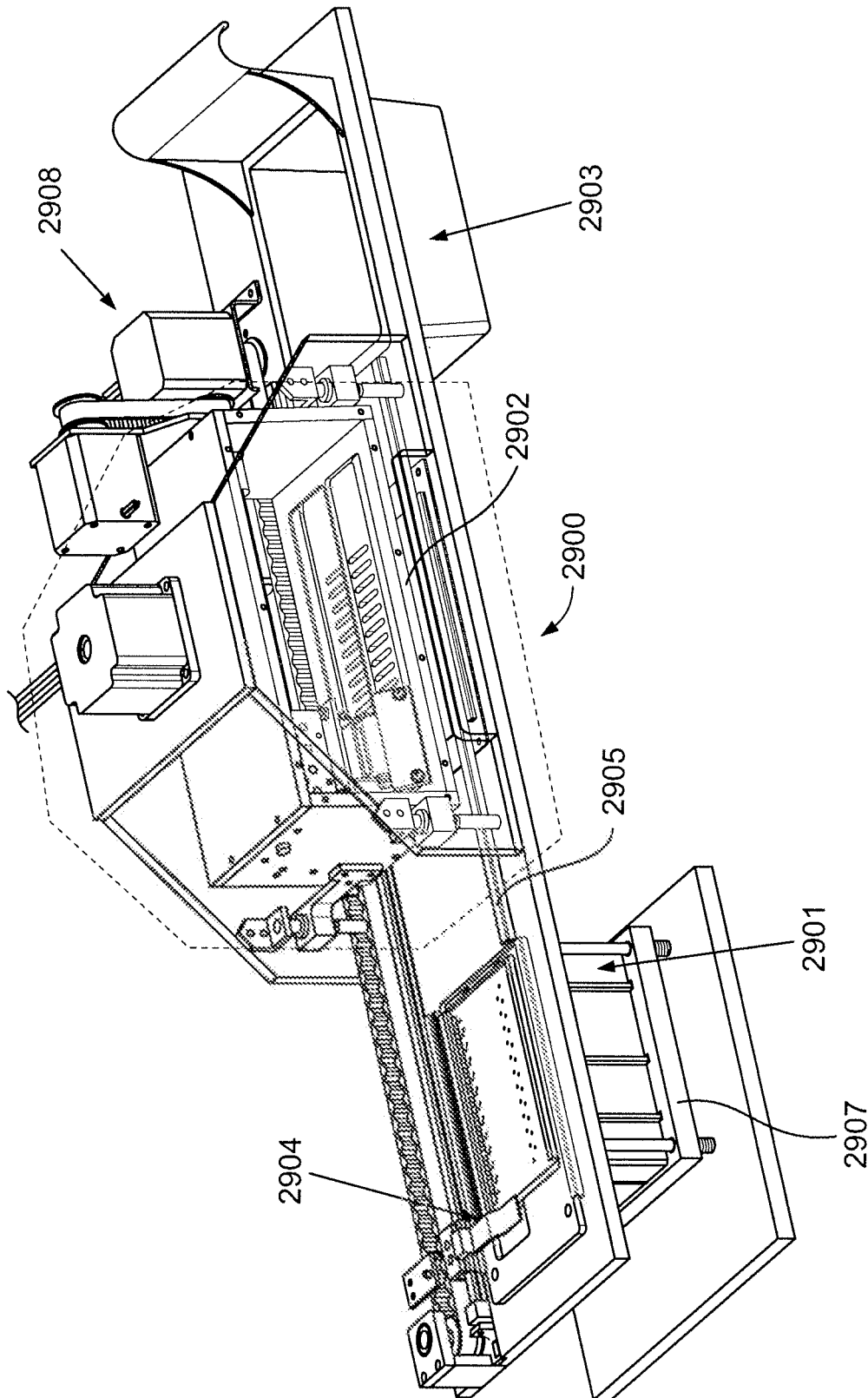


FIG. 28

U.S. Patent

Apr. 28, 2020

Sheet 47 of 121

US 10,632,466 B1

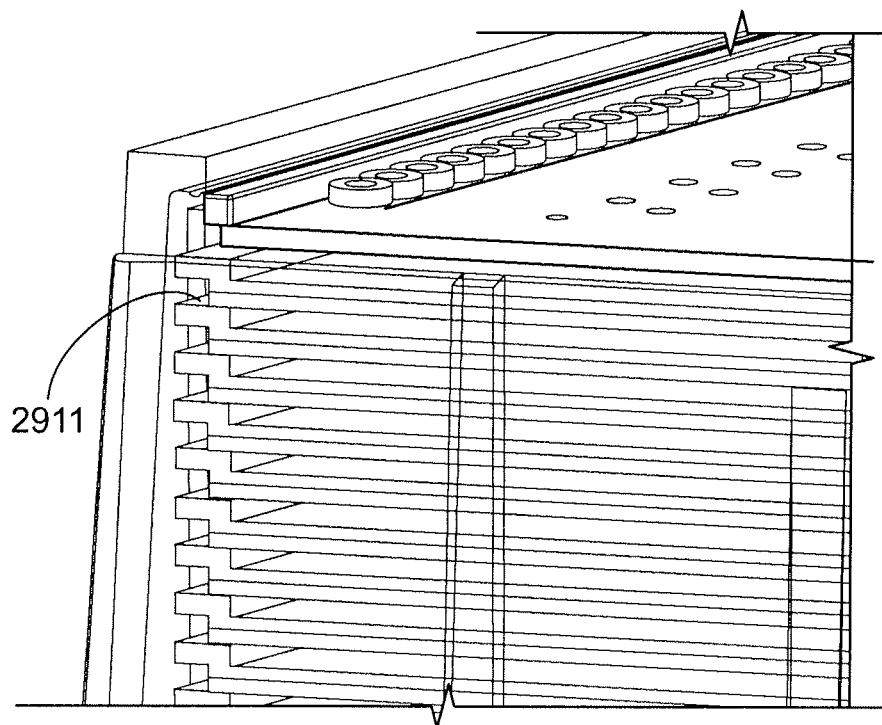
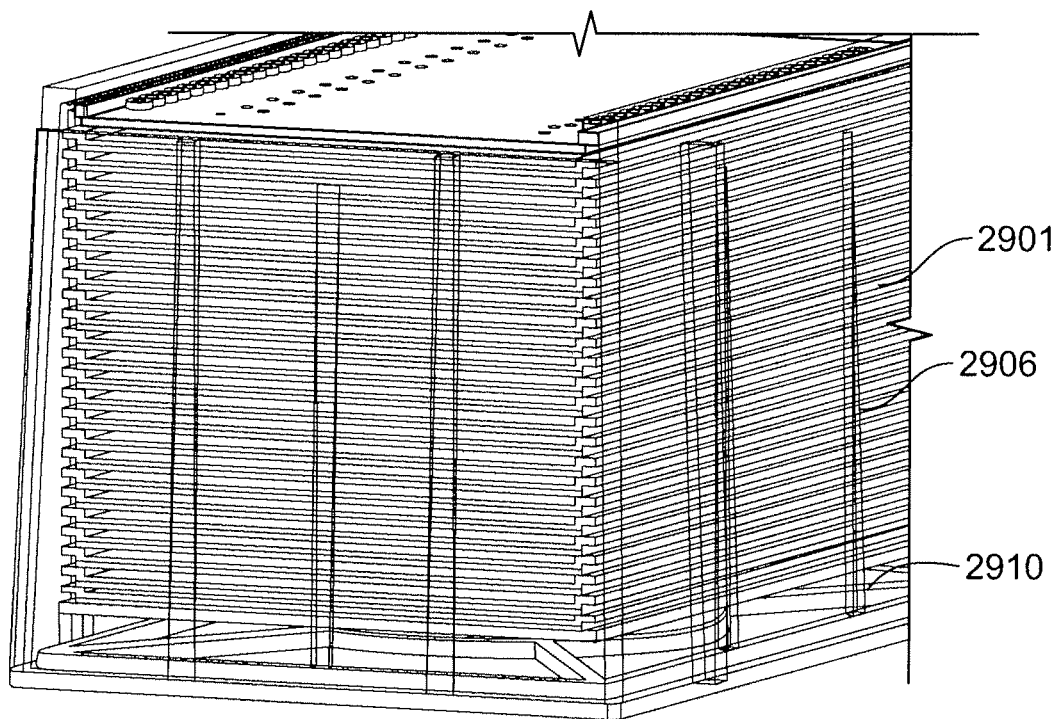


FIG. 29

U.S. Patent

Apr. 28, 2020

Sheet 48 of 121

US 10,632,466 B1

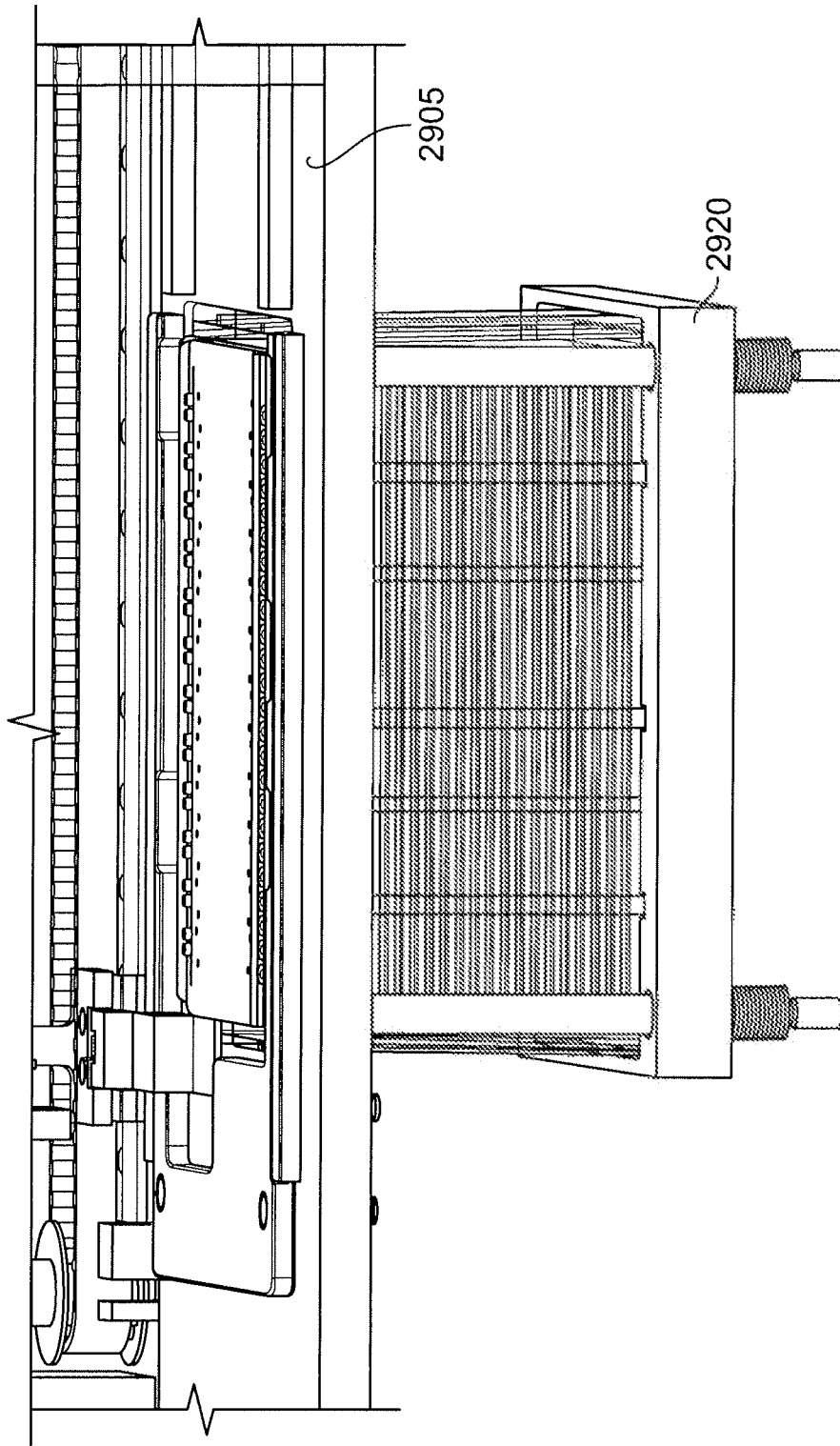


FIG. 30

U.S. Patent

Apr. 28, 2020

Sheet 49 of 121

US 10,632,466 B1

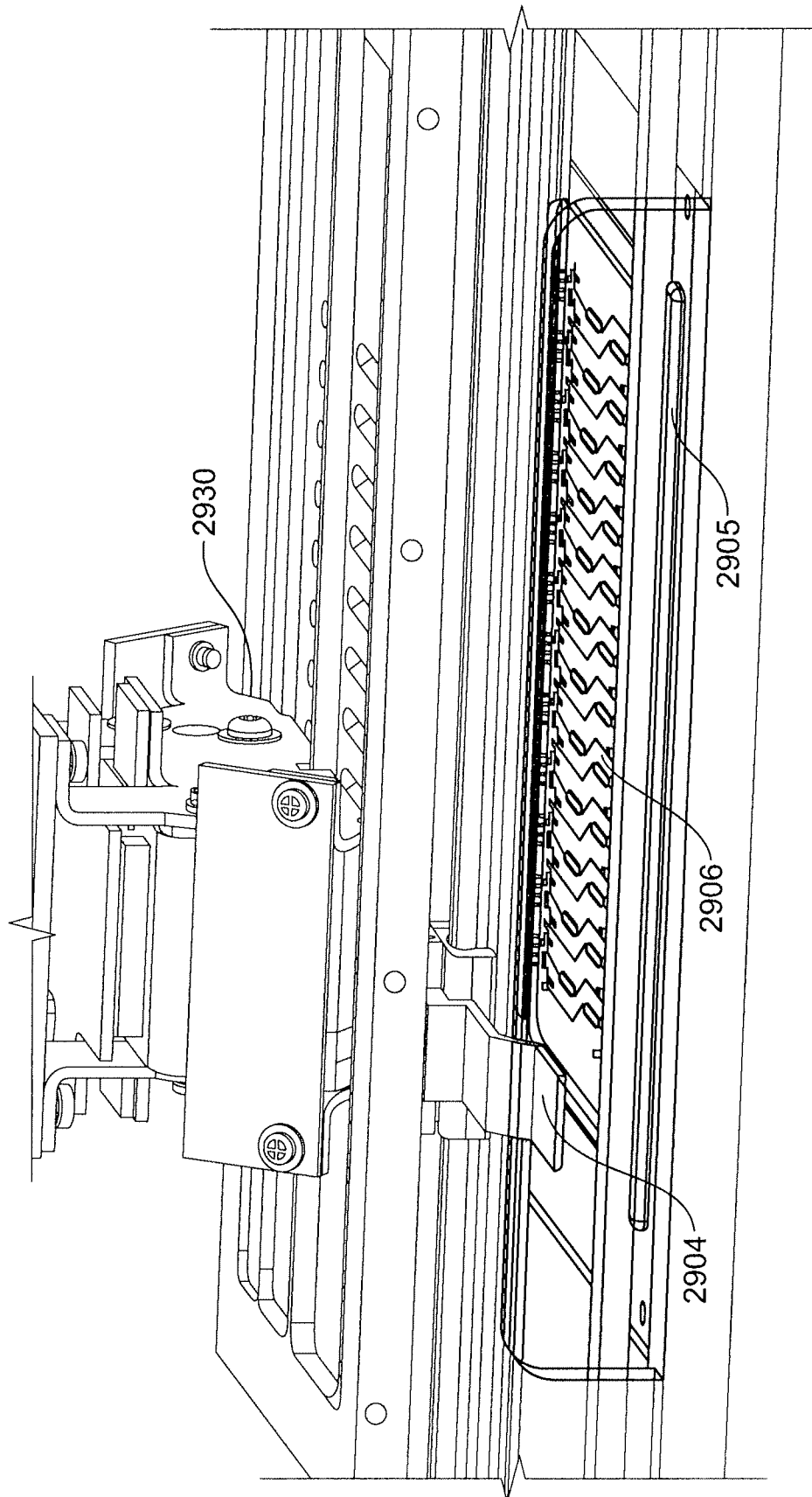


FIG. 31

U.S. Patent

Apr. 28, 2020

Sheet 50 of 121

US 10,632,466 B1

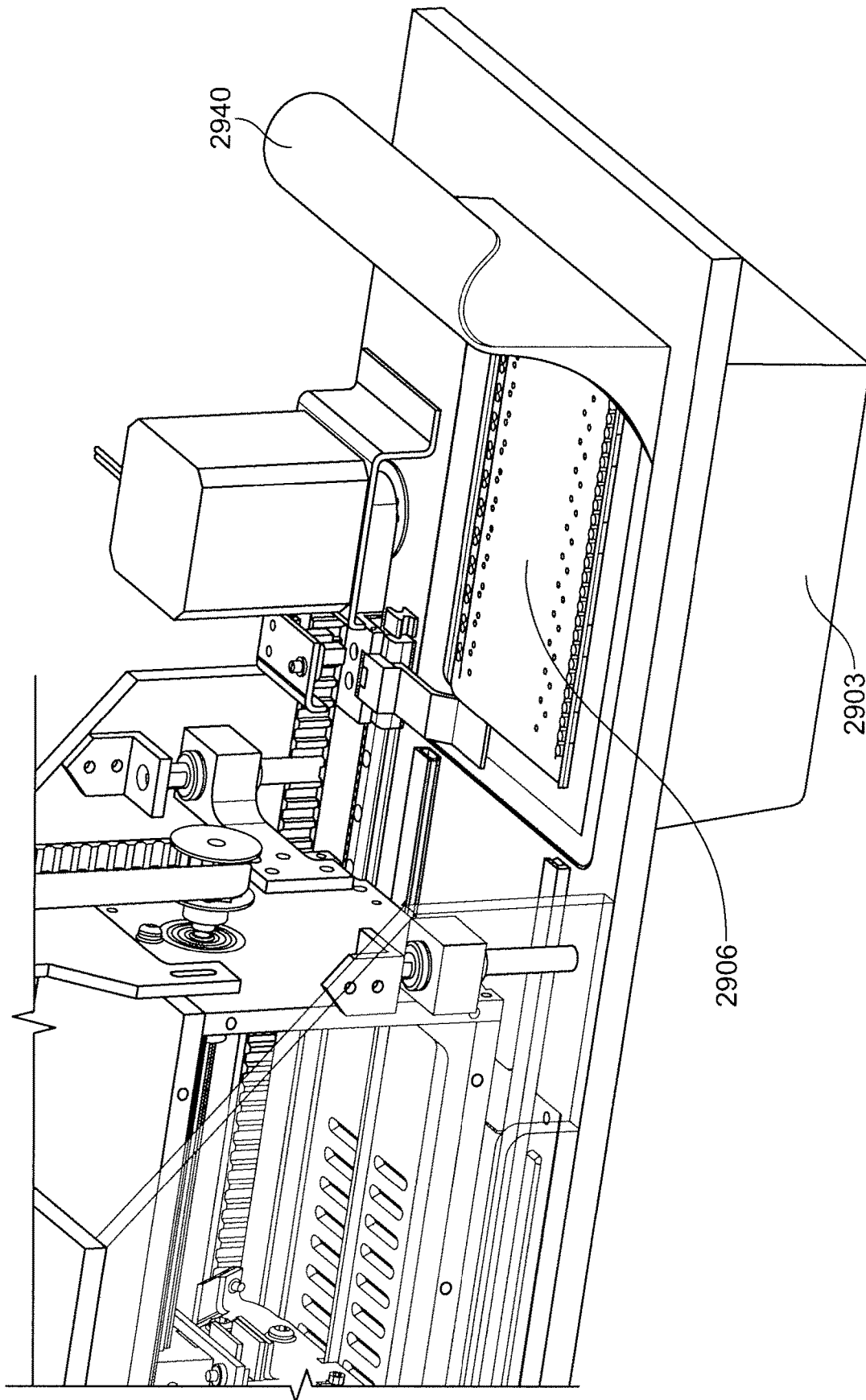


FIG. 32

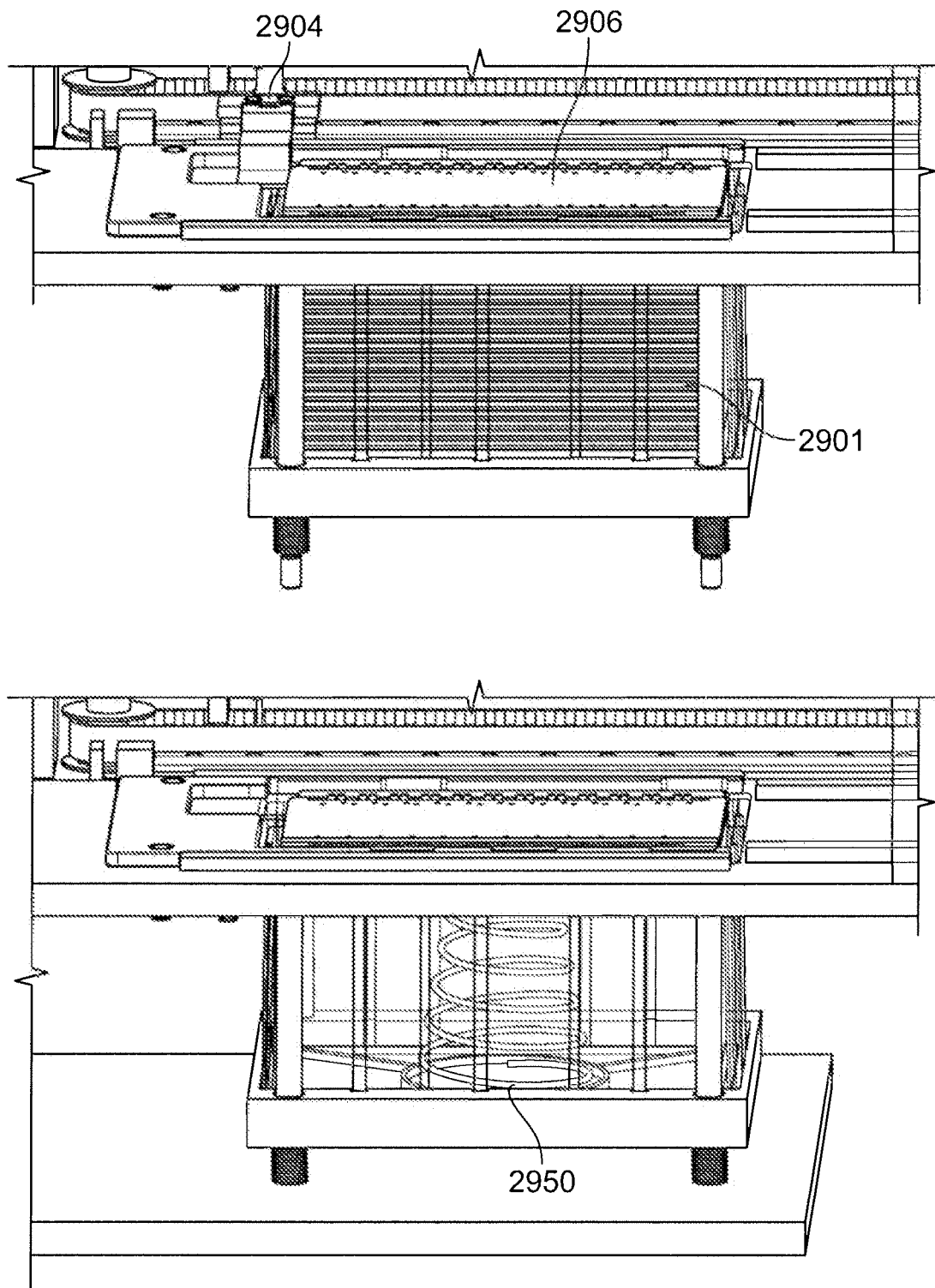


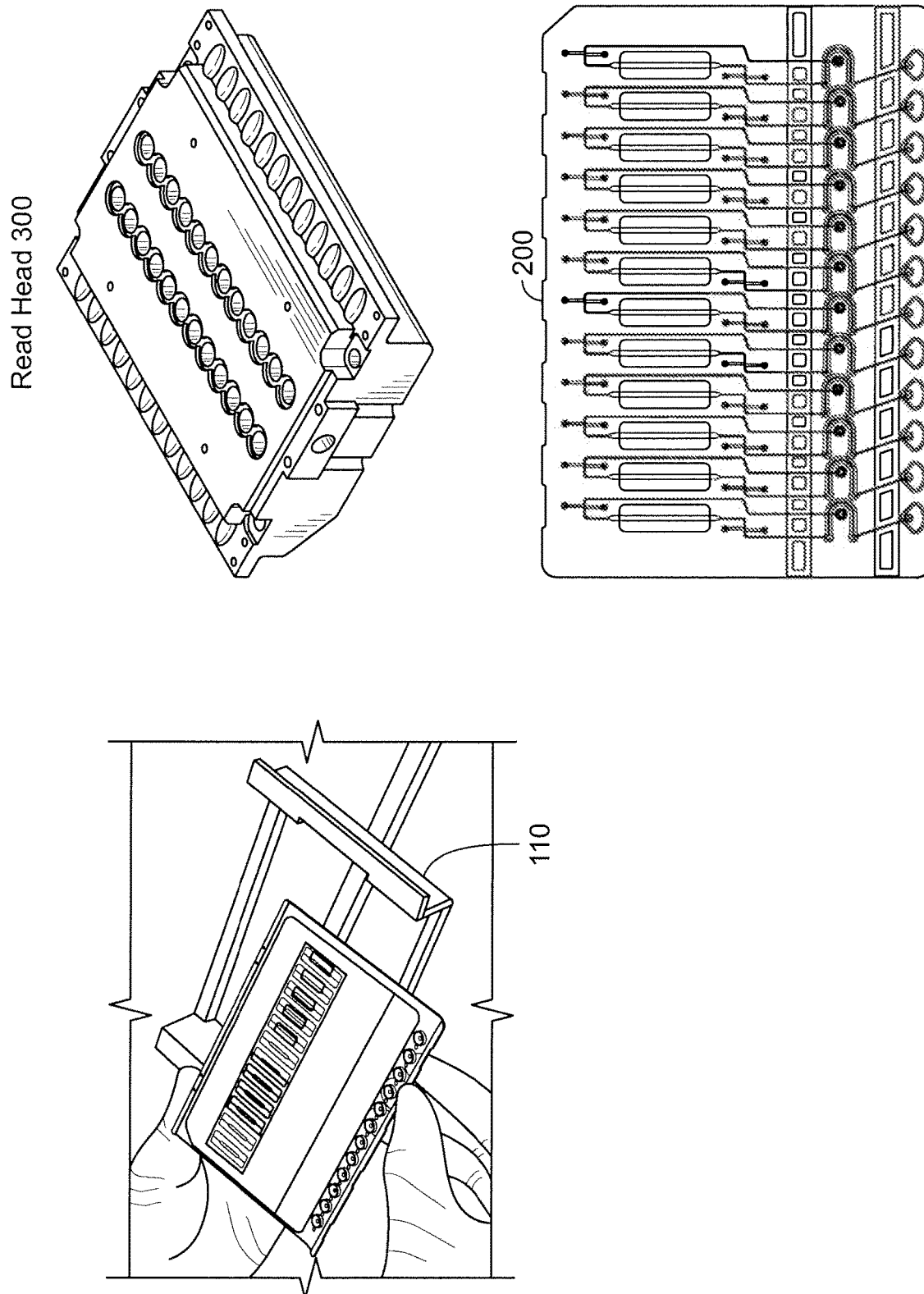
FIG. 33

U.S. Patent

Apr. 28, 2020

Sheet 52 of 121

US 10,632,466 B1



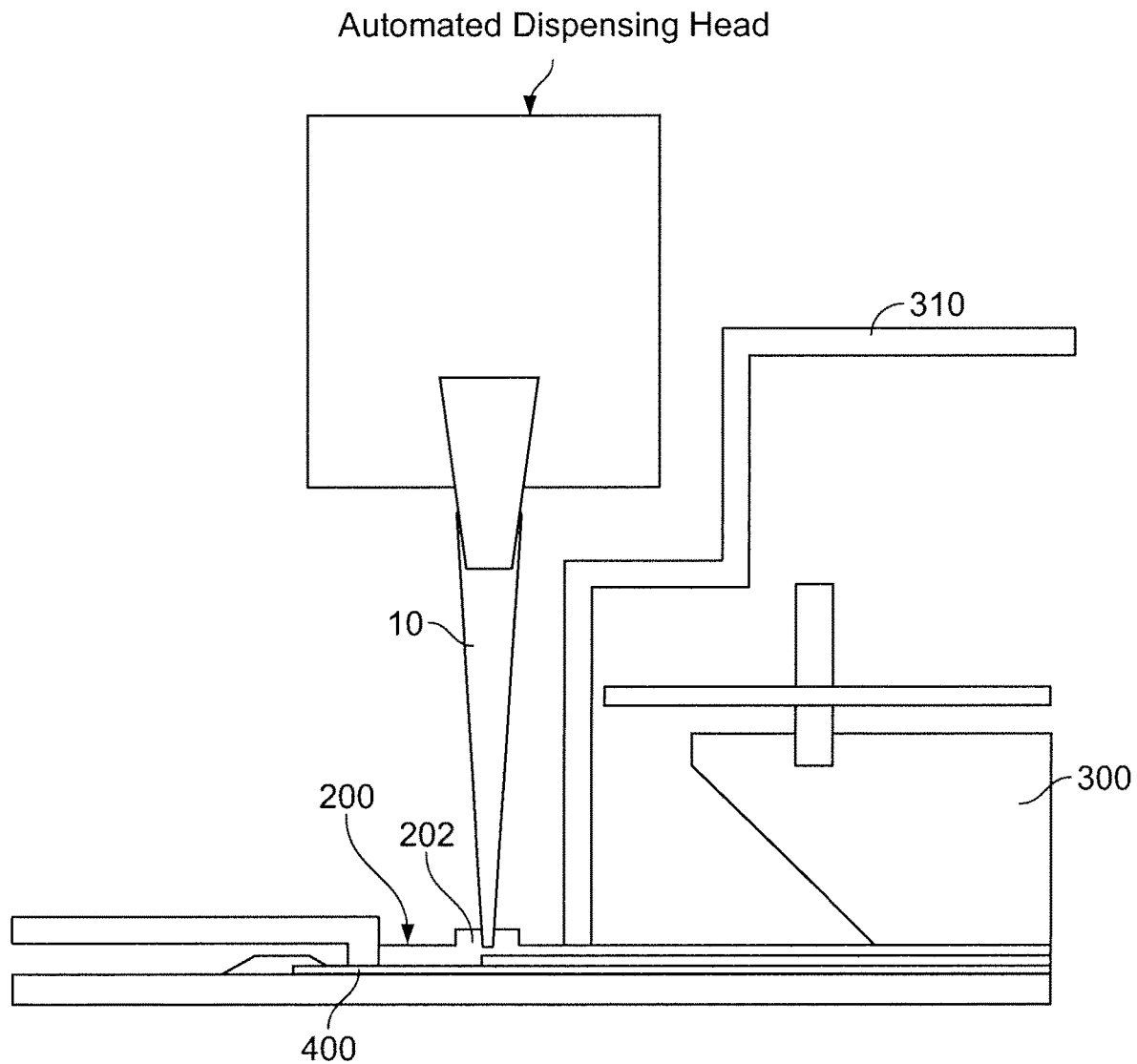


FIG. 35

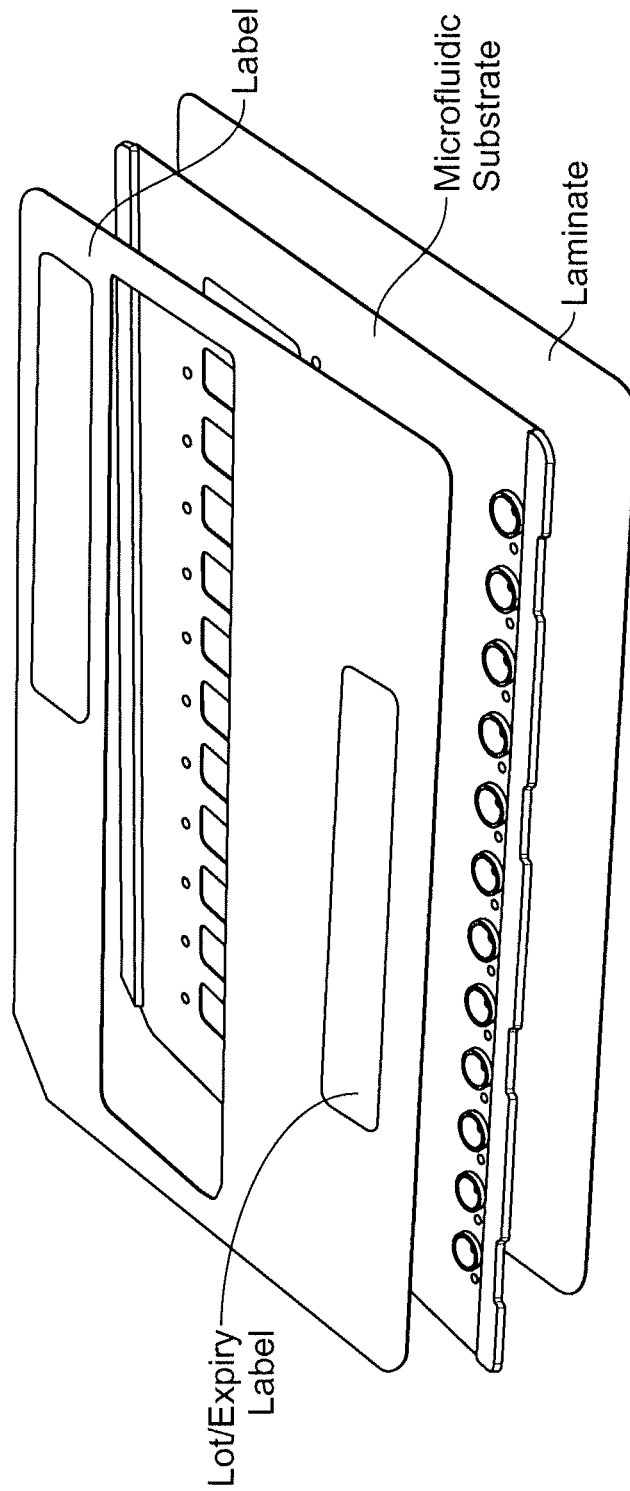


FIG. 36

U.S. Patent

Apr. 28, 2020

Sheet 55 of 121

US 10,632,466 B1

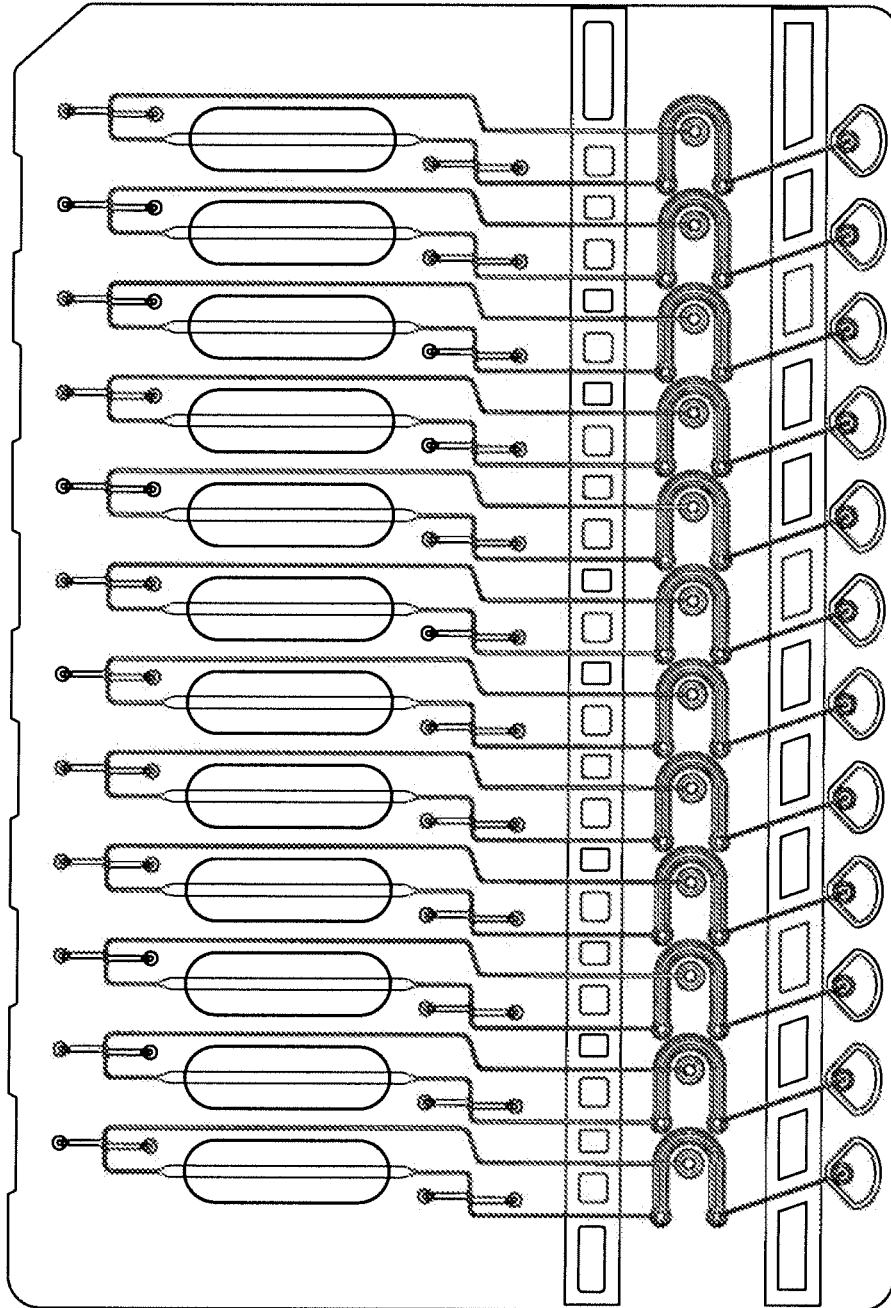


FIG. 37

U.S. Patent

Apr. 28, 2020

Sheet 56 of 121

US 10,632,466 B1

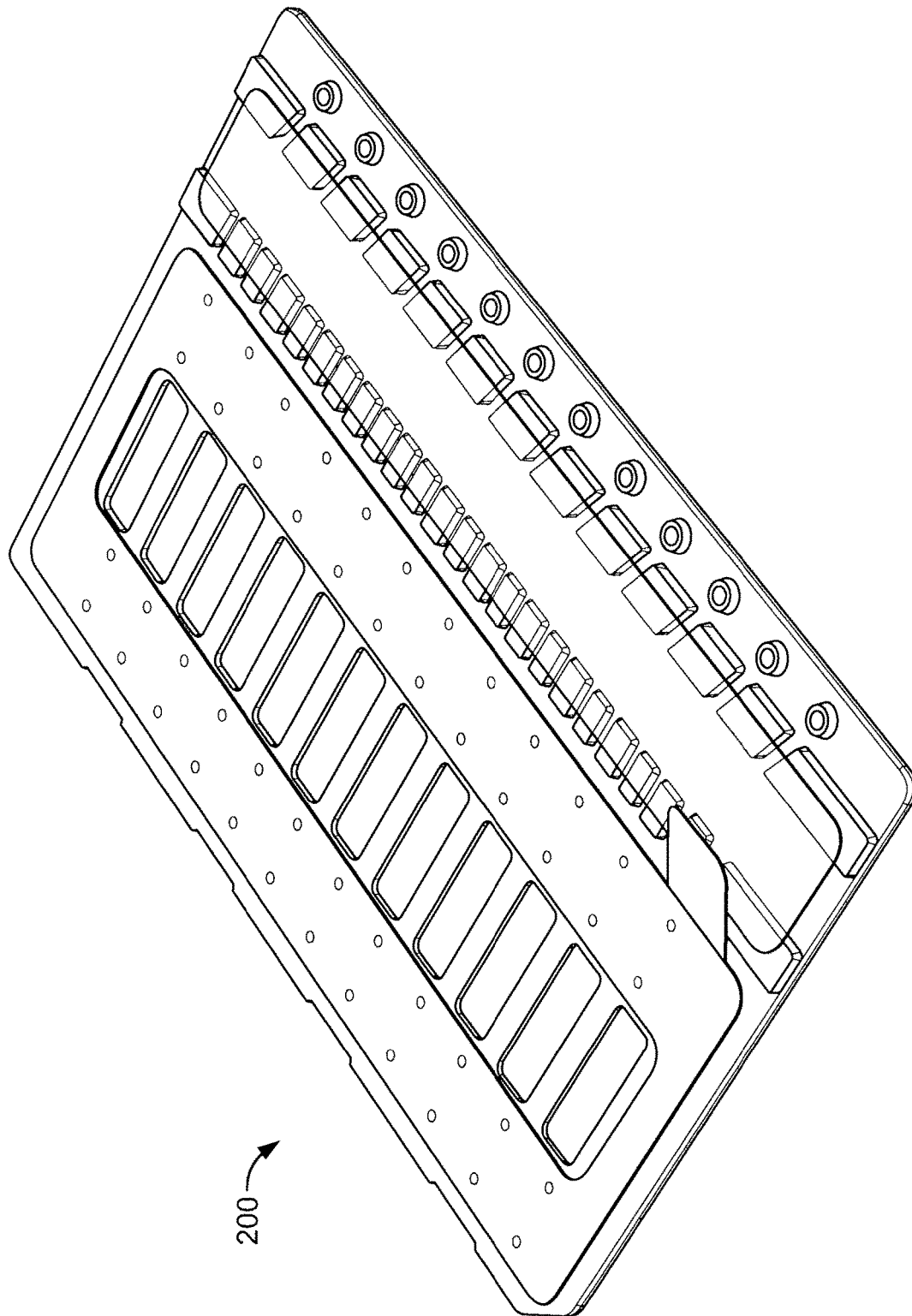


FIG. 38A

U.S. Patent

Apr. 28, 2020

Sheet 57 of 121

US 10,632,466 B1

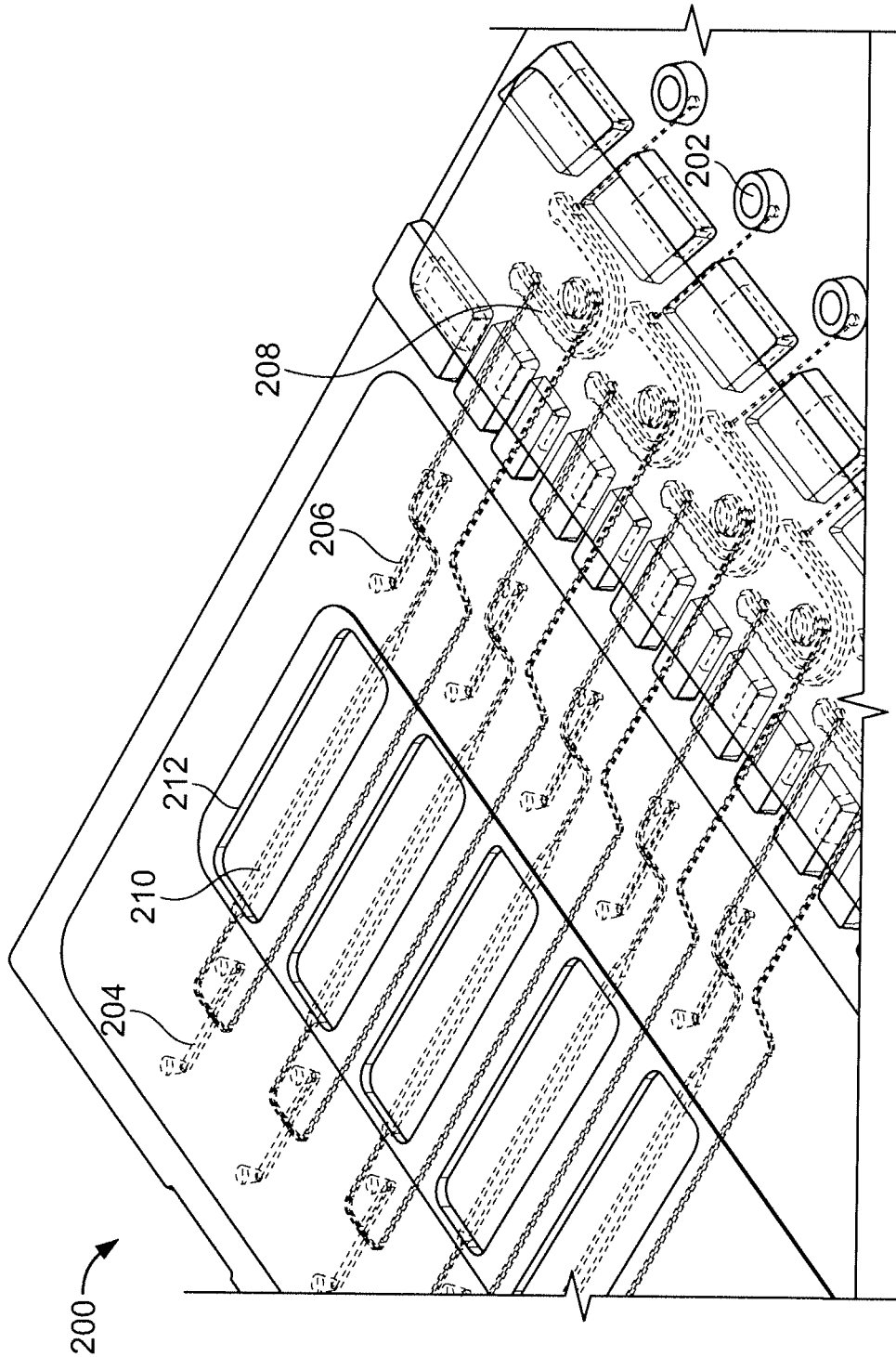


FIG. 38B

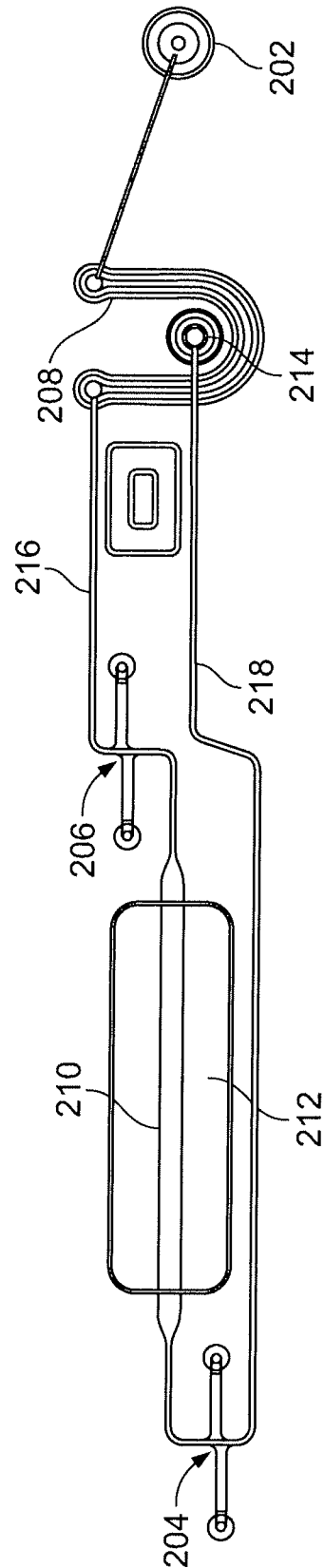


FIG. 39A

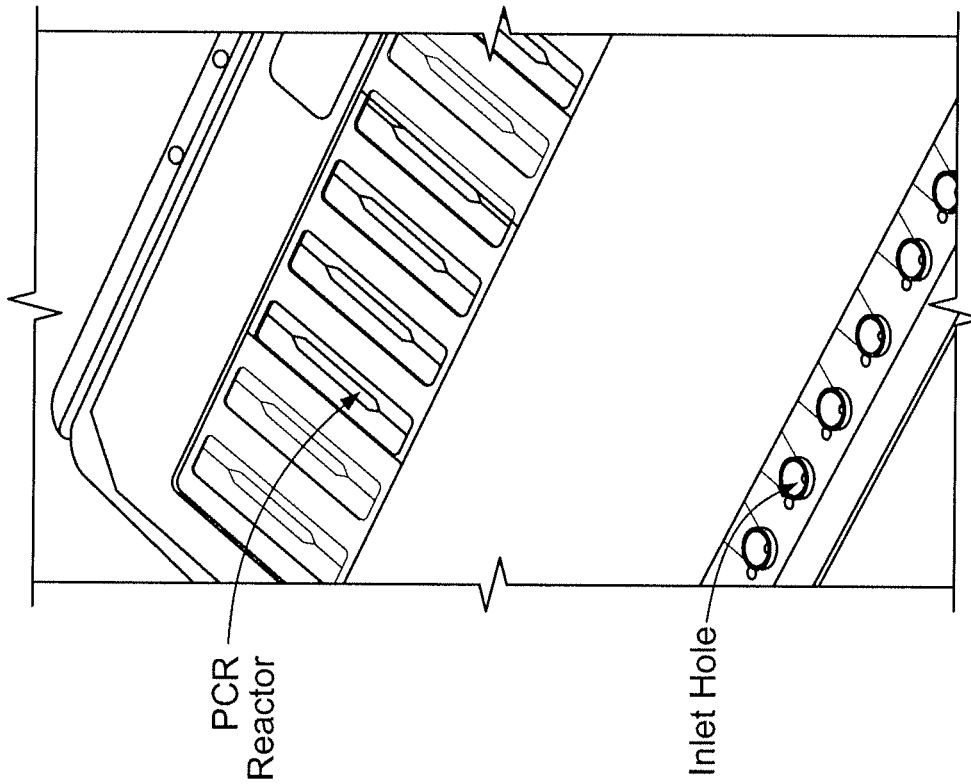
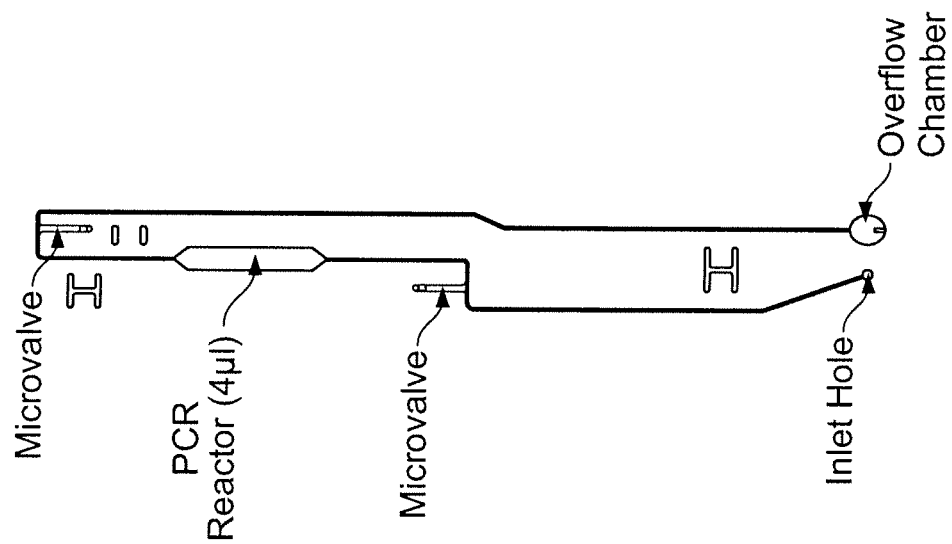


FIG. 39B



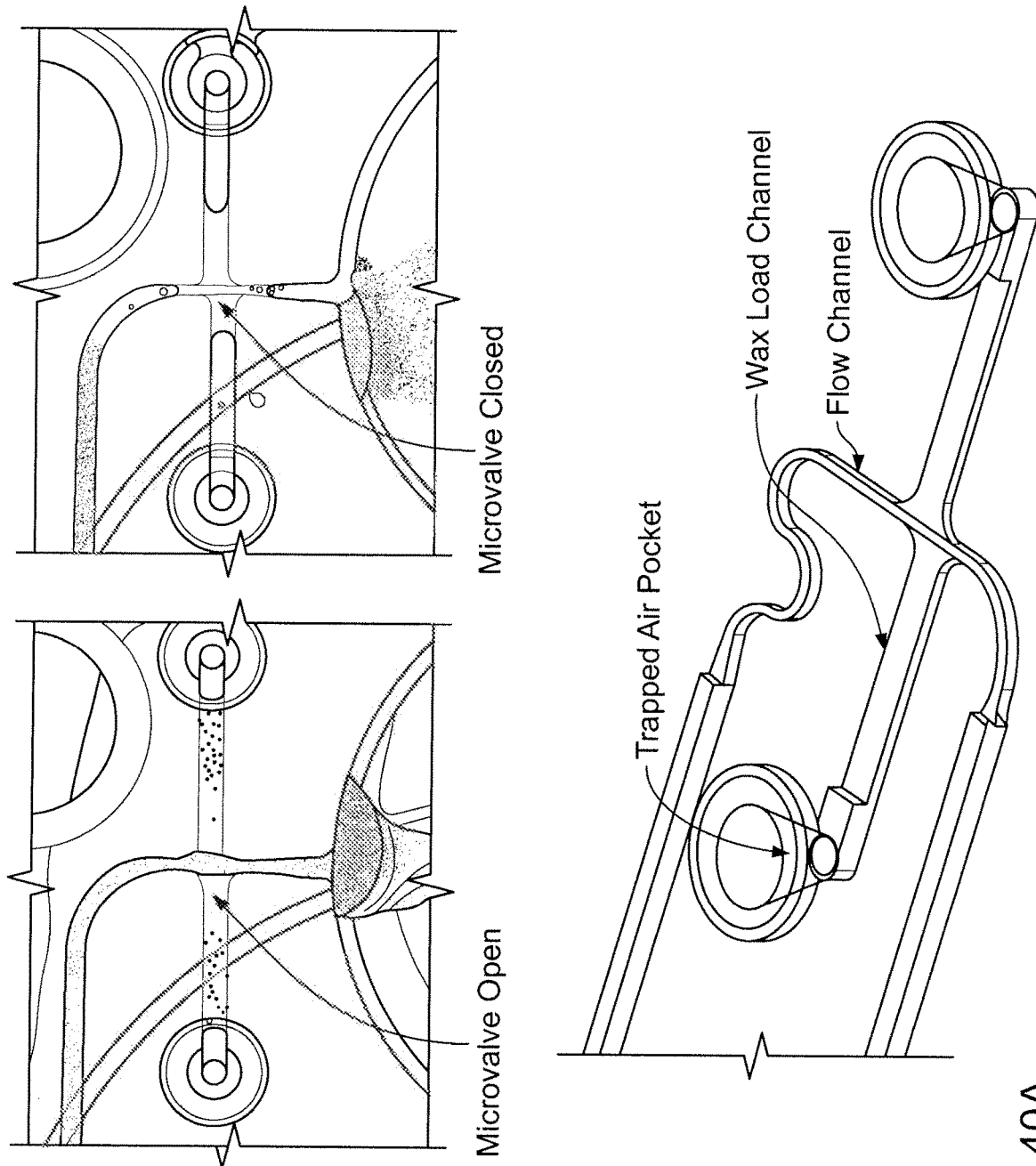


FIG. 40A

U.S. Patent

Apr. 28, 2020

Sheet 61 of 121

US 10,632,466 B1

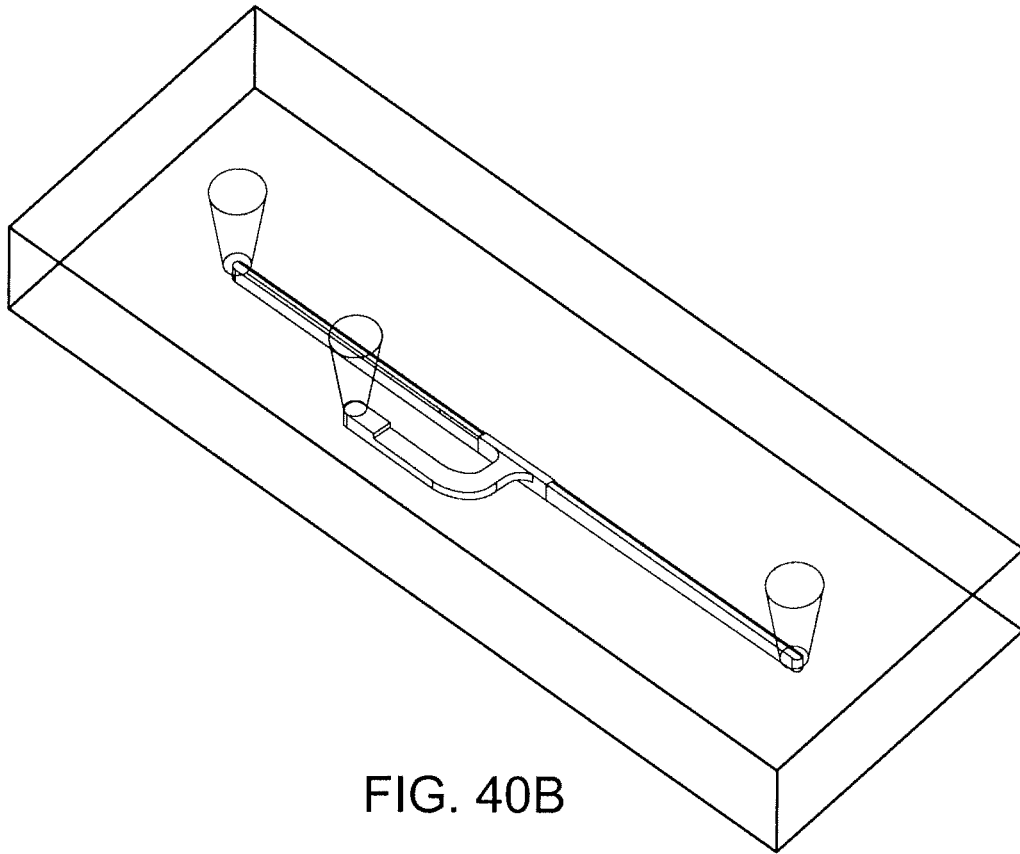


FIG. 40B

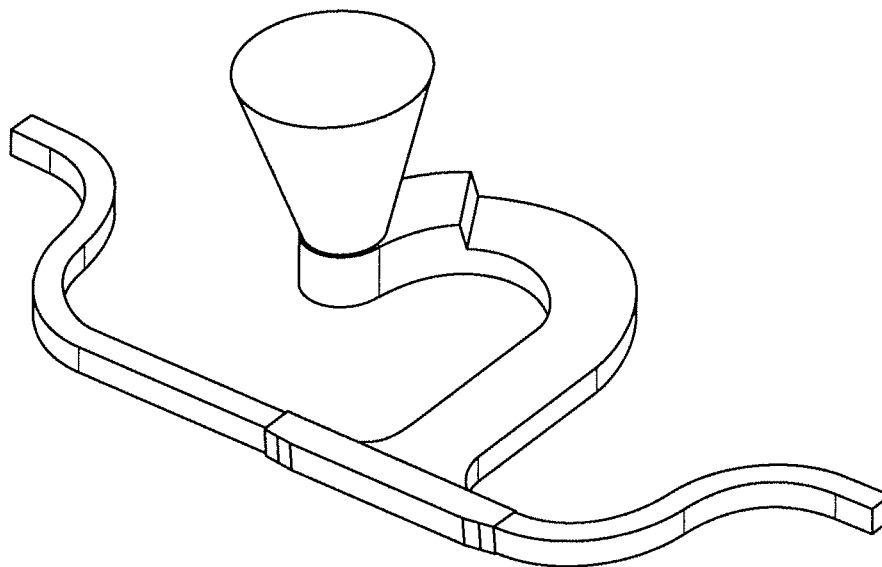


FIG. 40C

U.S. Patent

Apr. 28, 2020

Sheet 62 of 121

US 10,632,466 B1

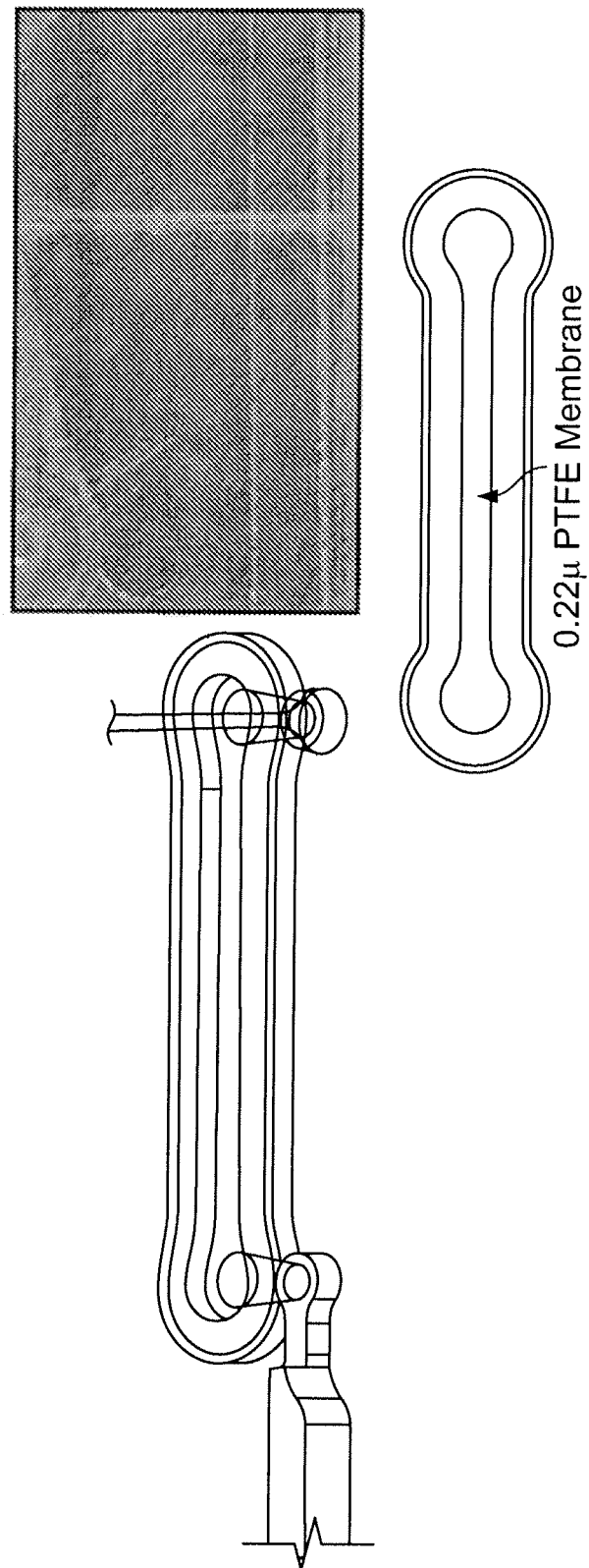


FIG. 41

U.S. Patent

Apr. 28, 2020

Sheet 63 of 121

US 10,632,466 B1

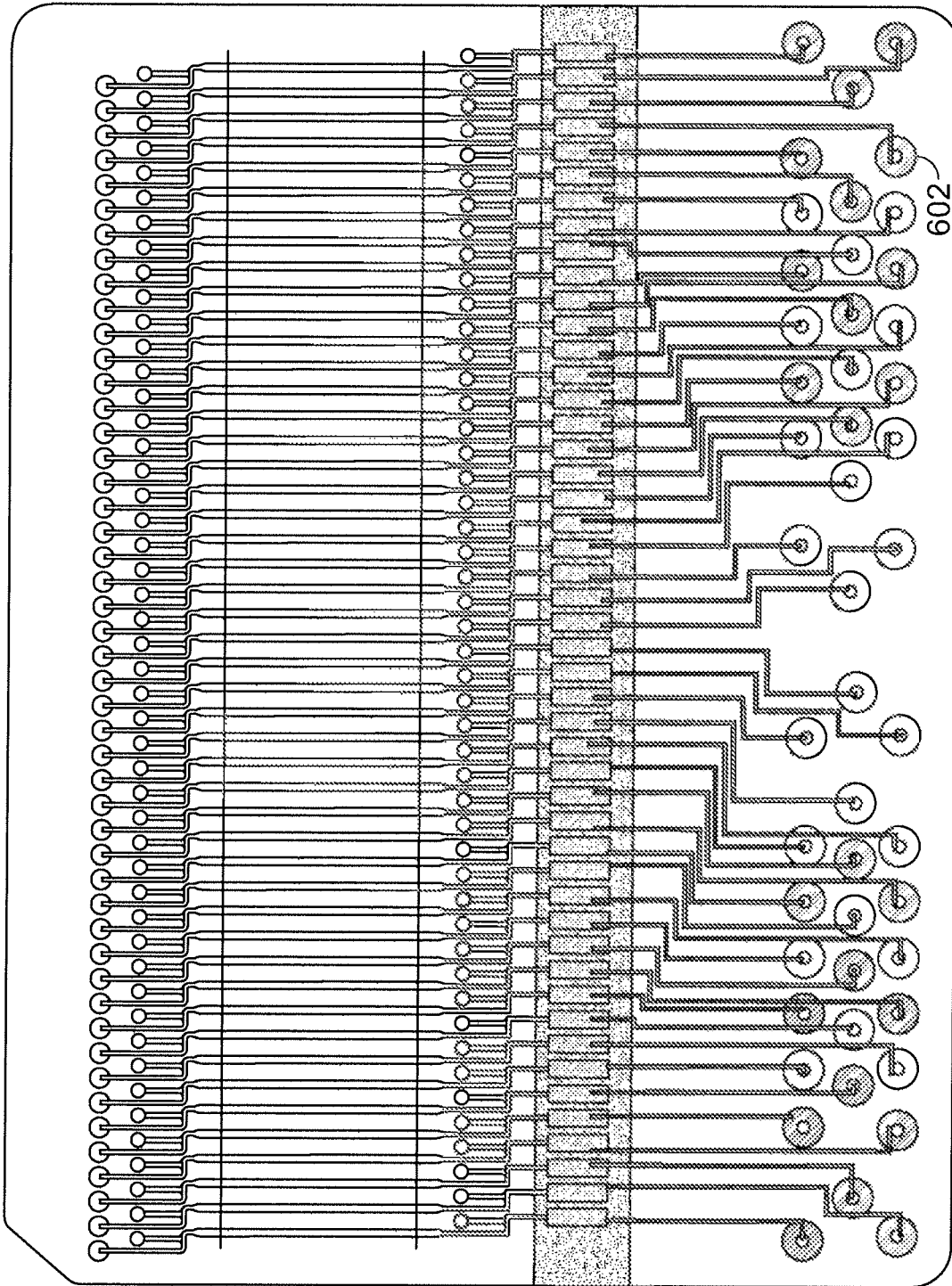


FIG. 42

U.S. Patent

Apr. 28, 2020

Sheet 64 of 121

US 10,632,466 B1

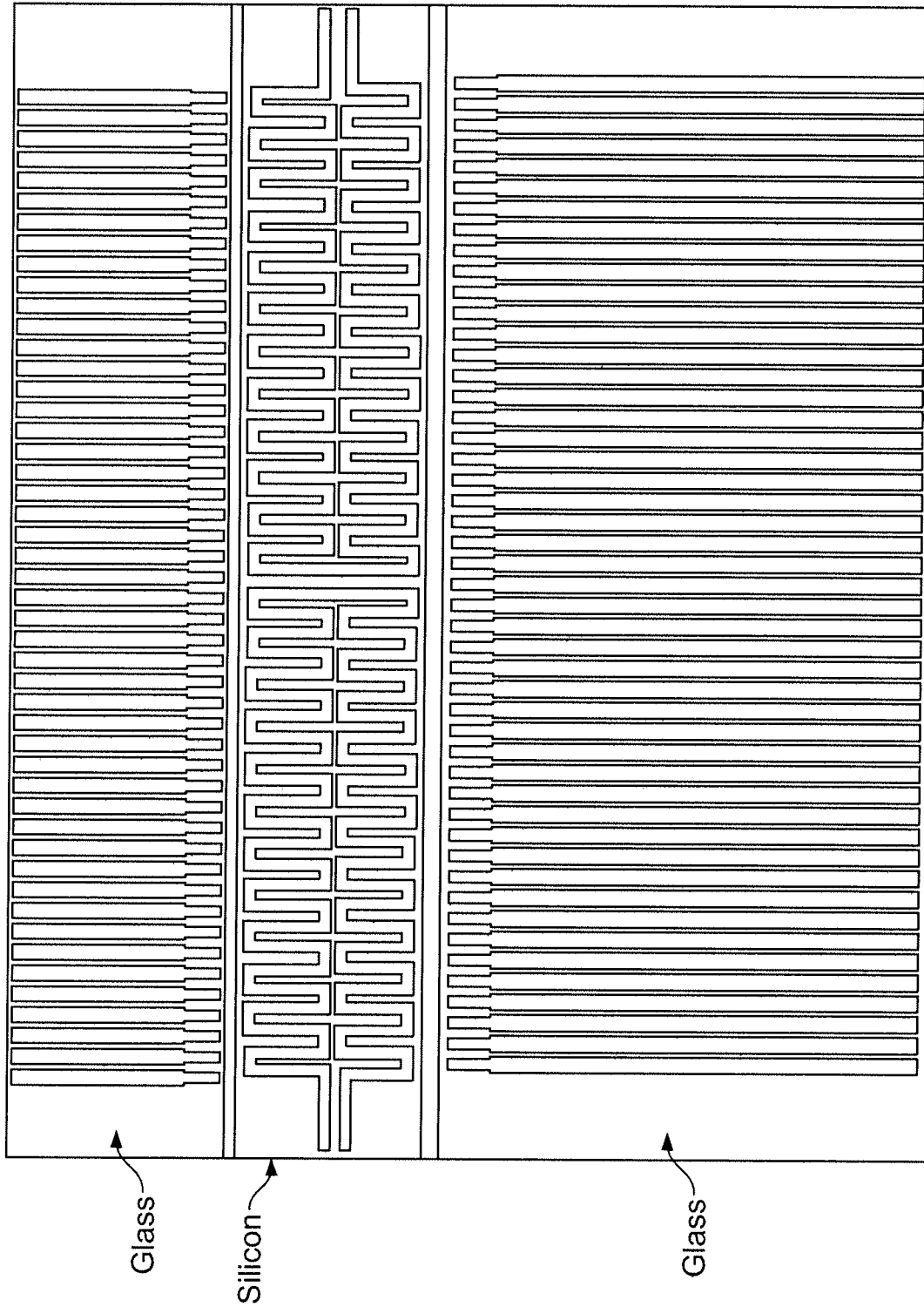


FIG. 43

U.S. Patent

Apr. 28, 2020

Sheet 65 of 121

US 10,632,466 B1

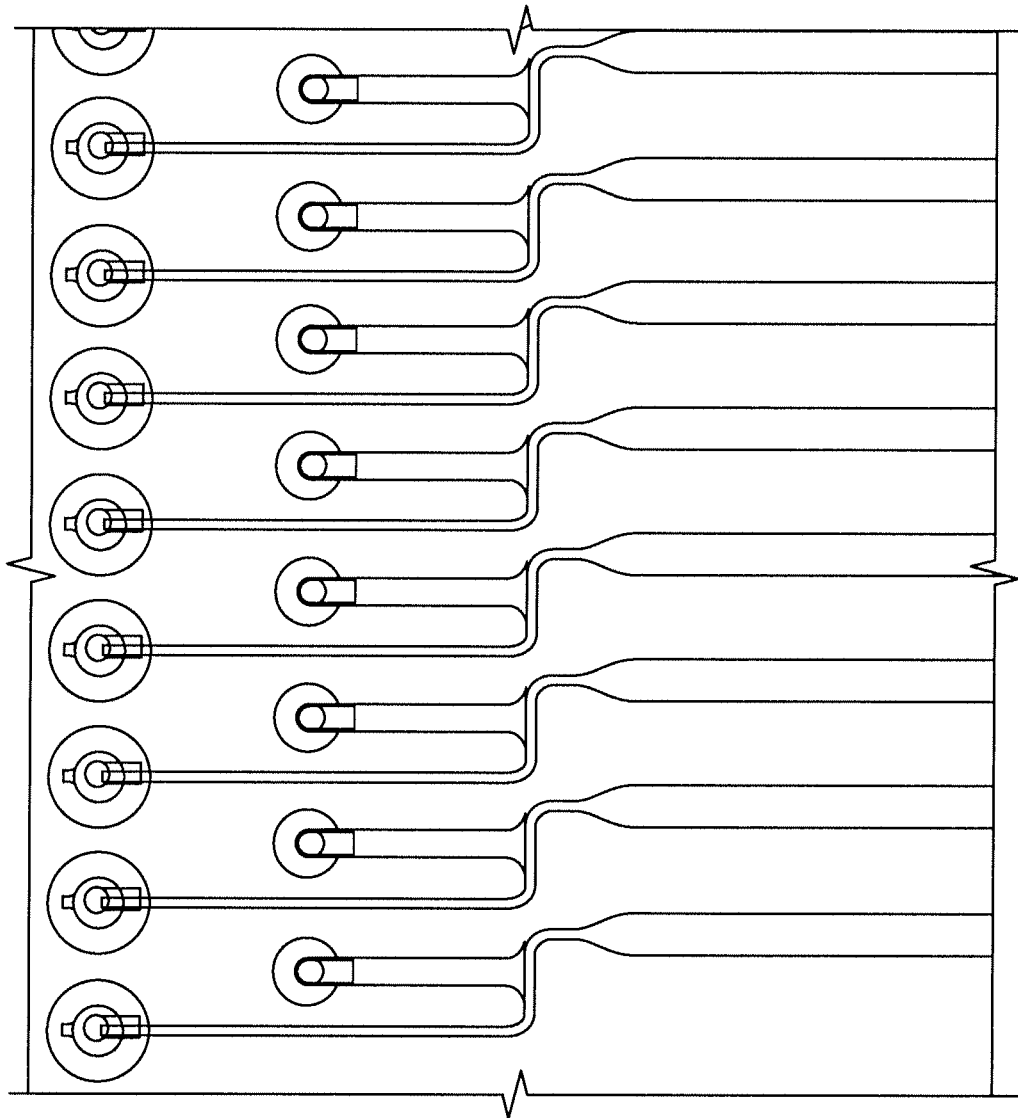


FIG. 44

U.S. Patent

Apr. 28, 2020

Sheet 66 of 121

US 10,632,466 B1

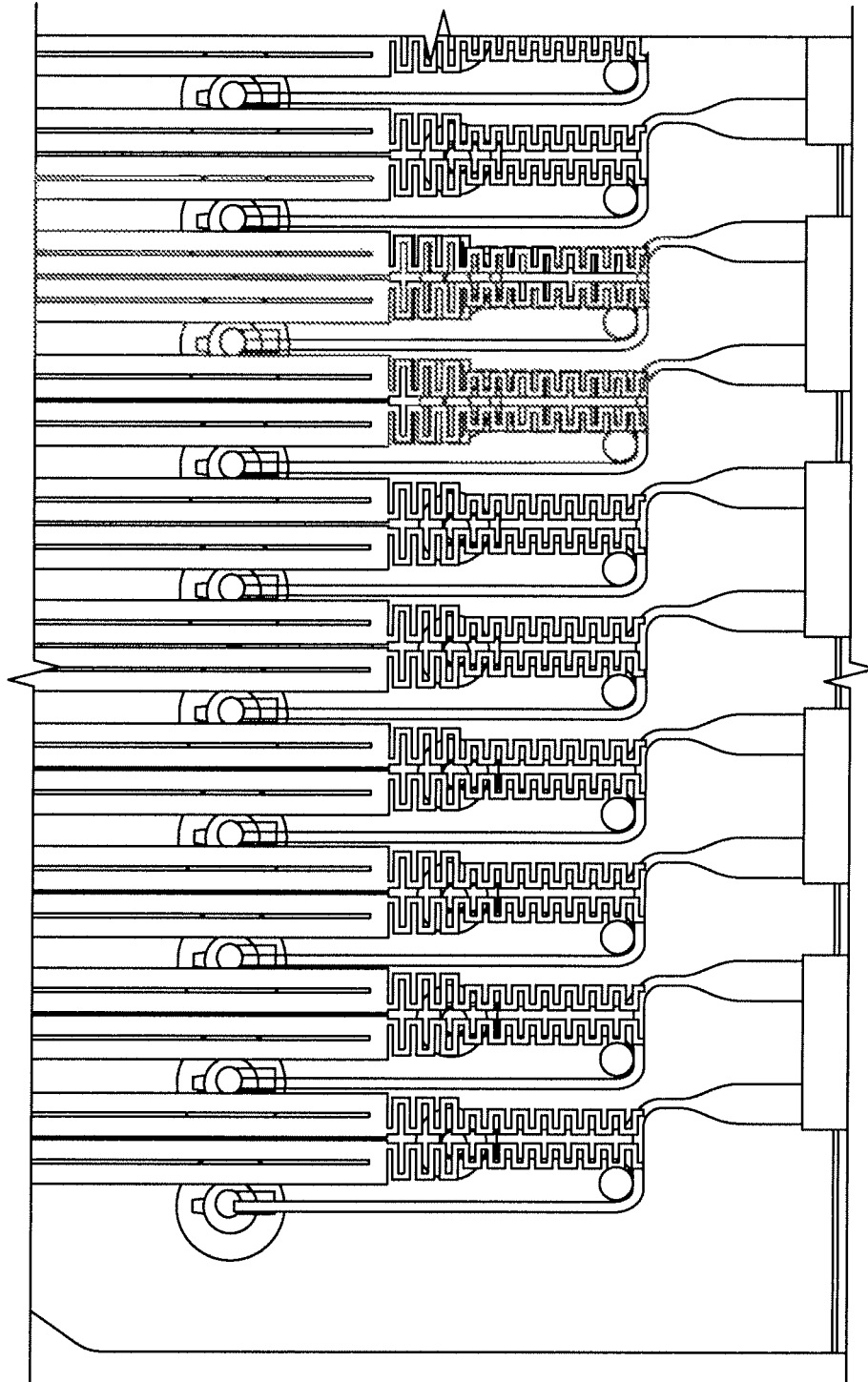


FIG. 45

U.S. Patent

Apr. 28, 2020

Sheet 67 of 121

US 10,632,466 B1

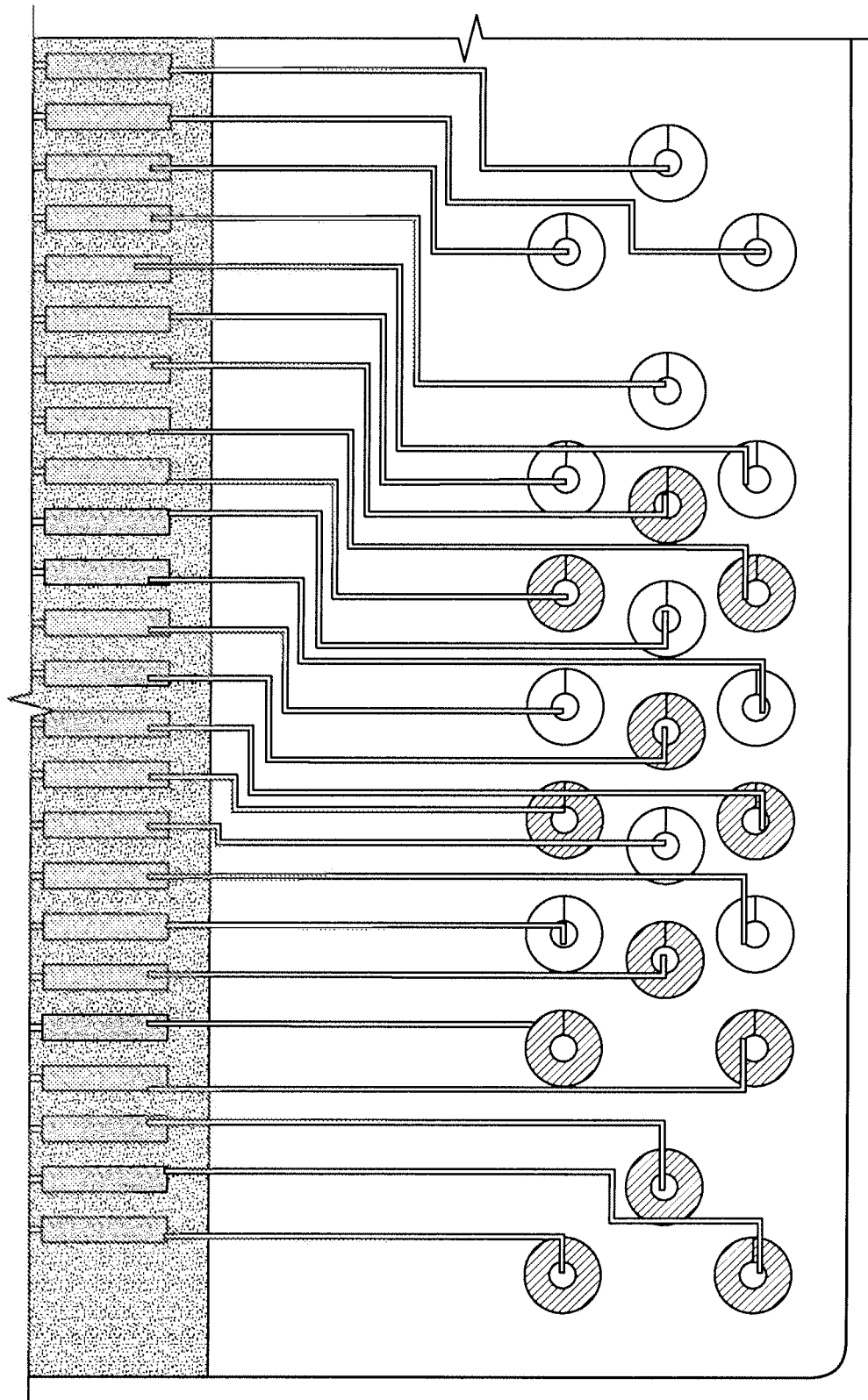


FIG. 46

U.S. Patent

Apr. 28, 2020

Sheet 68 of 121

US 10,632,466 B1

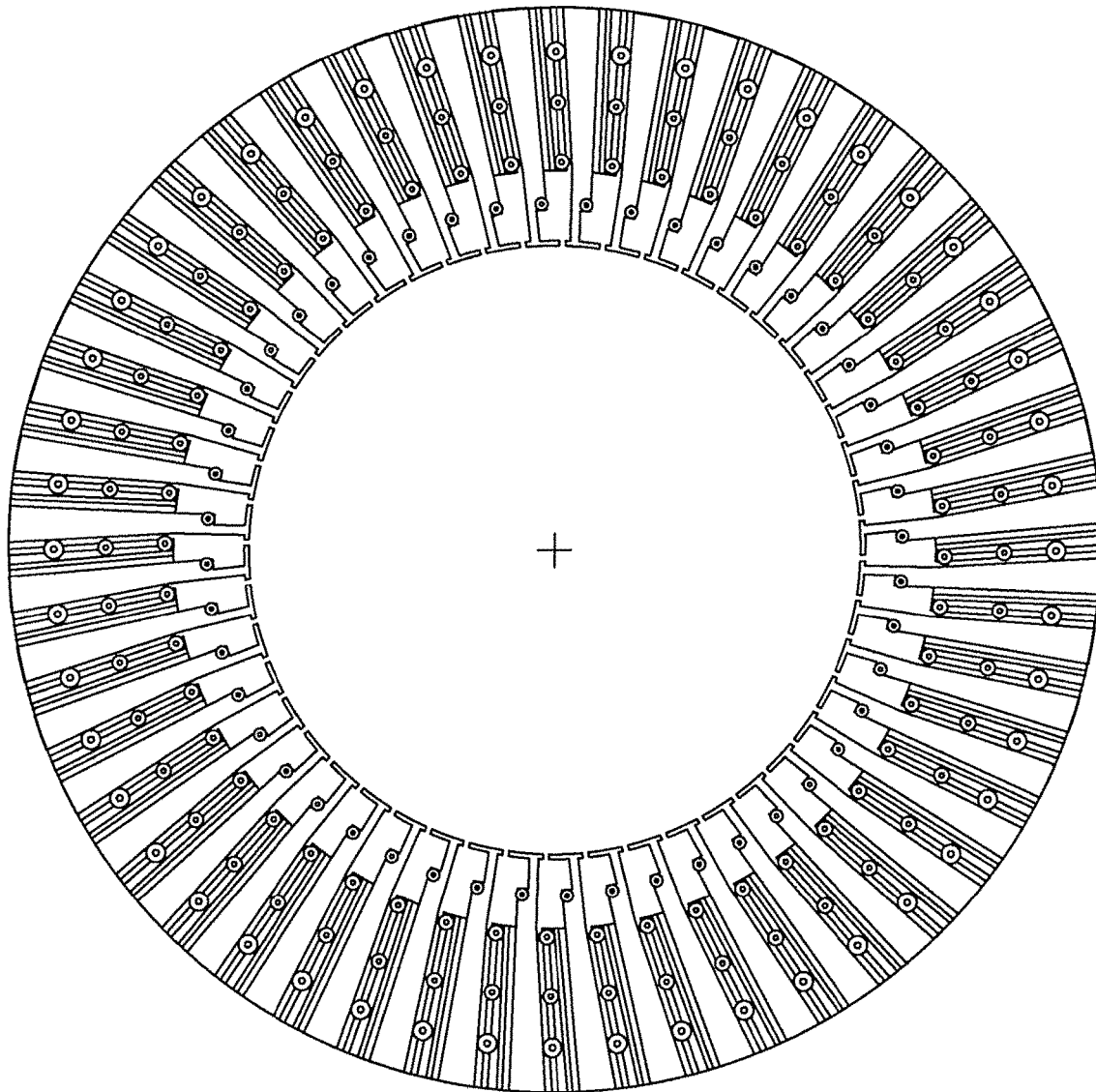


FIG. 47A

U.S. Patent

Apr. 28, 2020

Sheet 69 of 121

US 10,632,466 B1

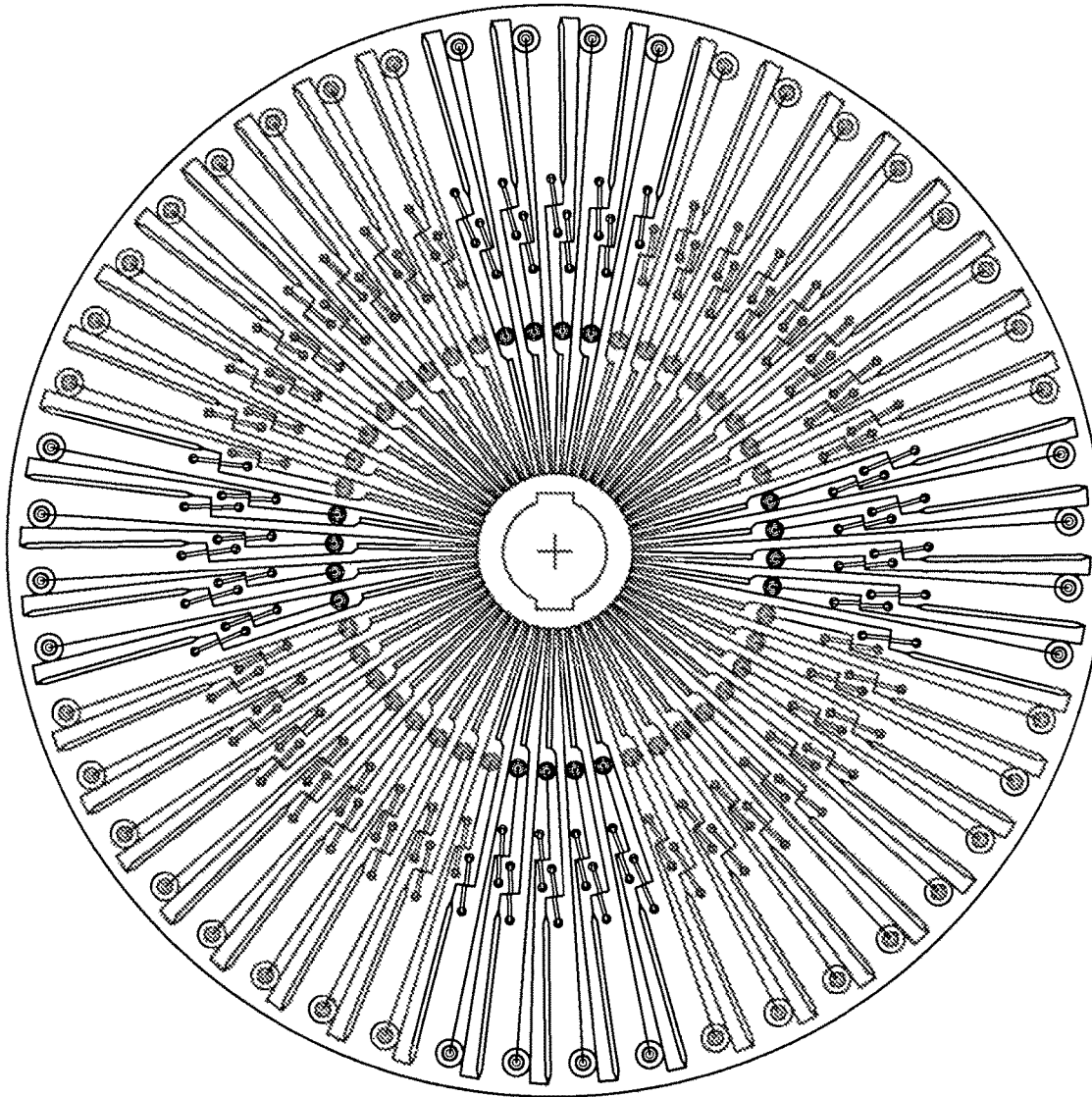


FIG. 47B

U.S. Patent

Apr. 28, 2020

Sheet 70 of 121

US 10,632,466 B1

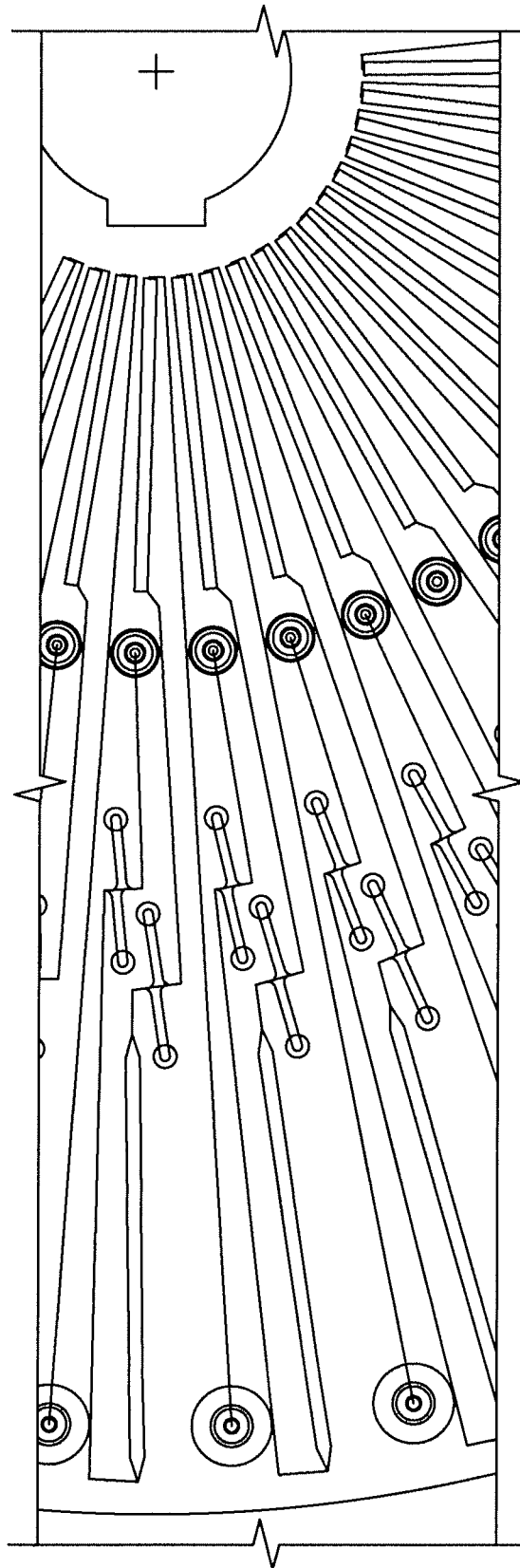


FIG. 47C

U.S. Patent

Apr. 28, 2020

Sheet 71 of 121

US 10,632,466 B1

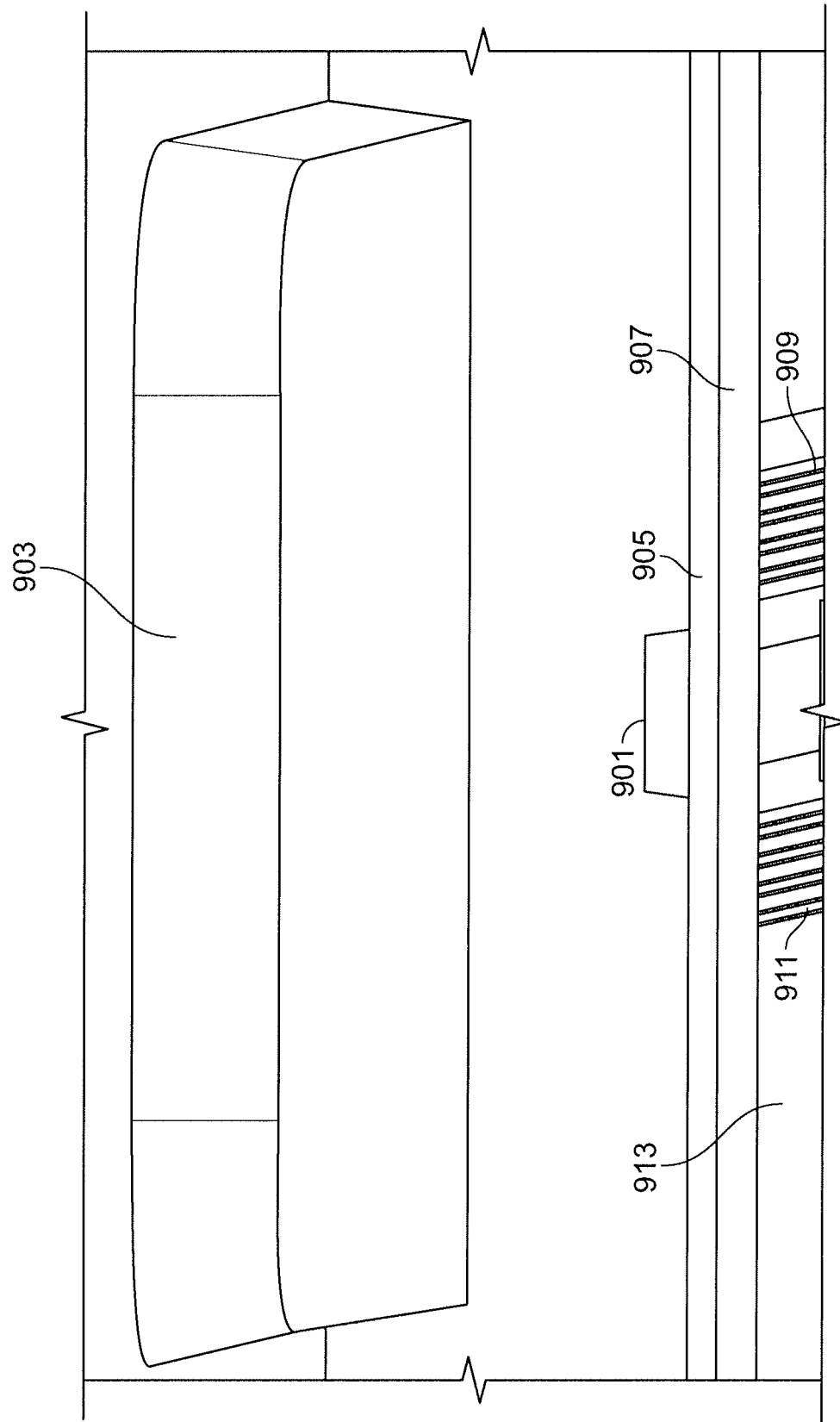


FIG. 48

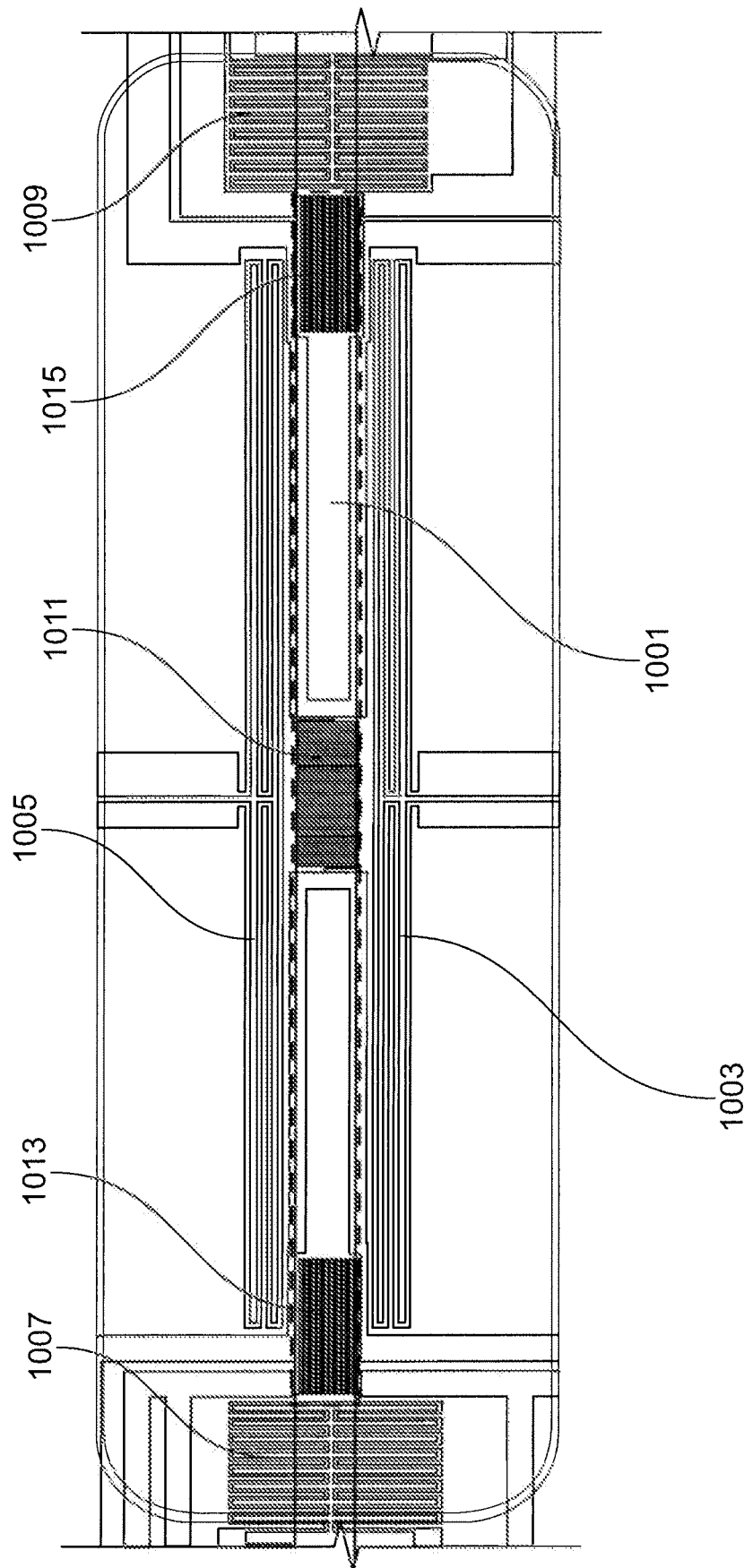


FIG. 49A

U.S. Patent

Apr. 28, 2020

Sheet 73 of 121

US 10,632,466 B1

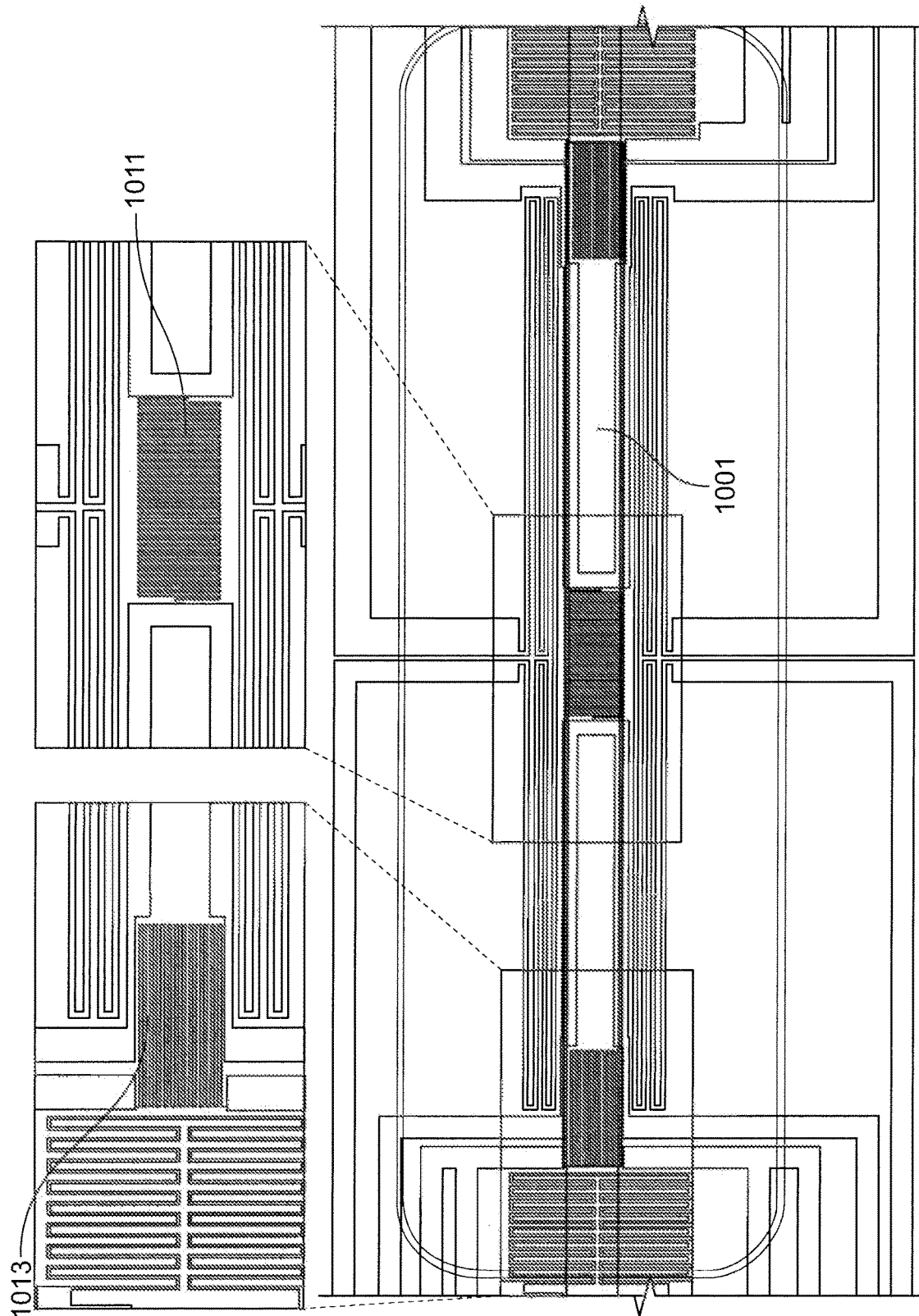


FIG. 49B

U.S. Patent

Apr. 28, 2020

Sheet 74 of 121

US 10,632,466 B1

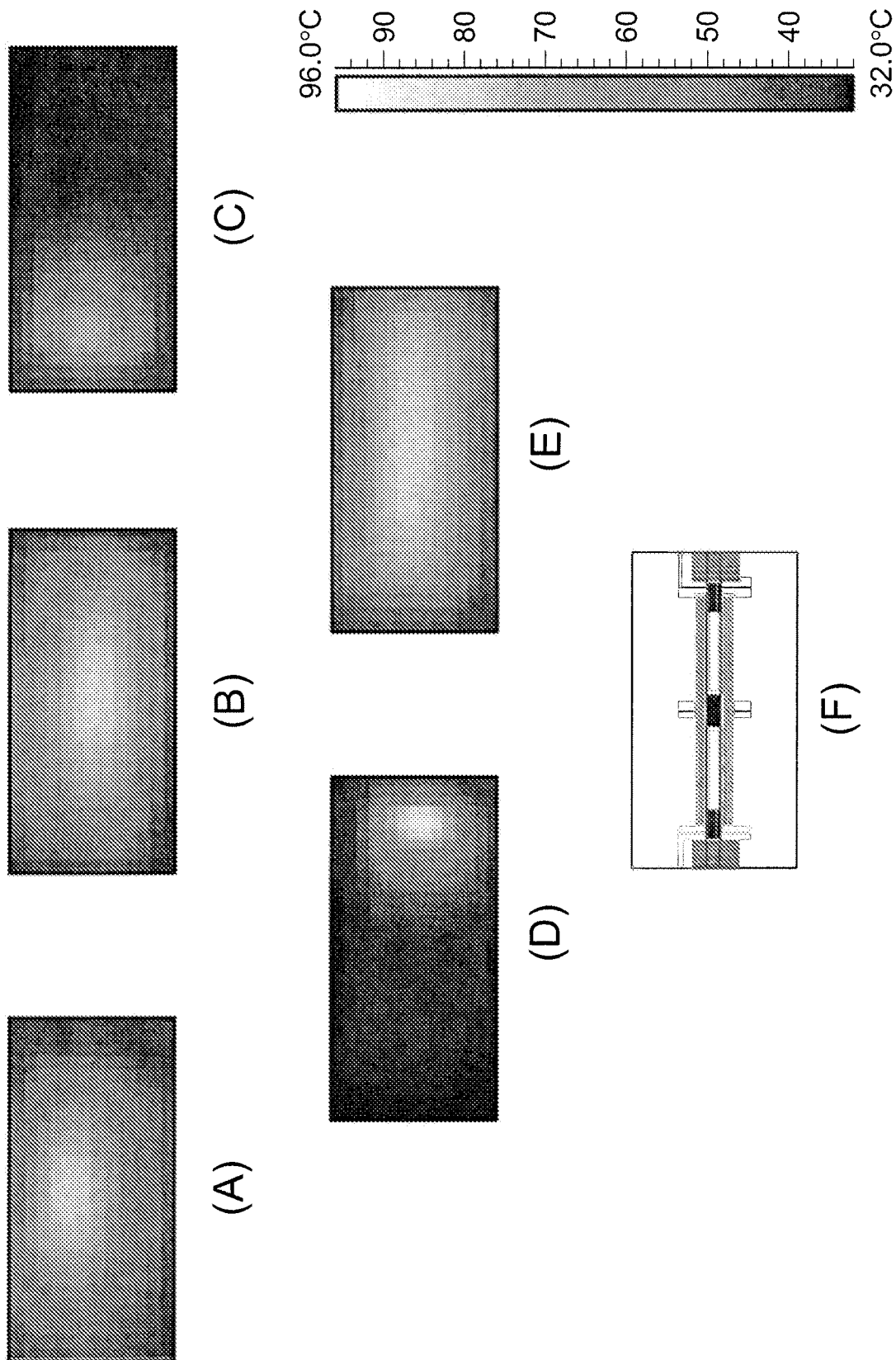
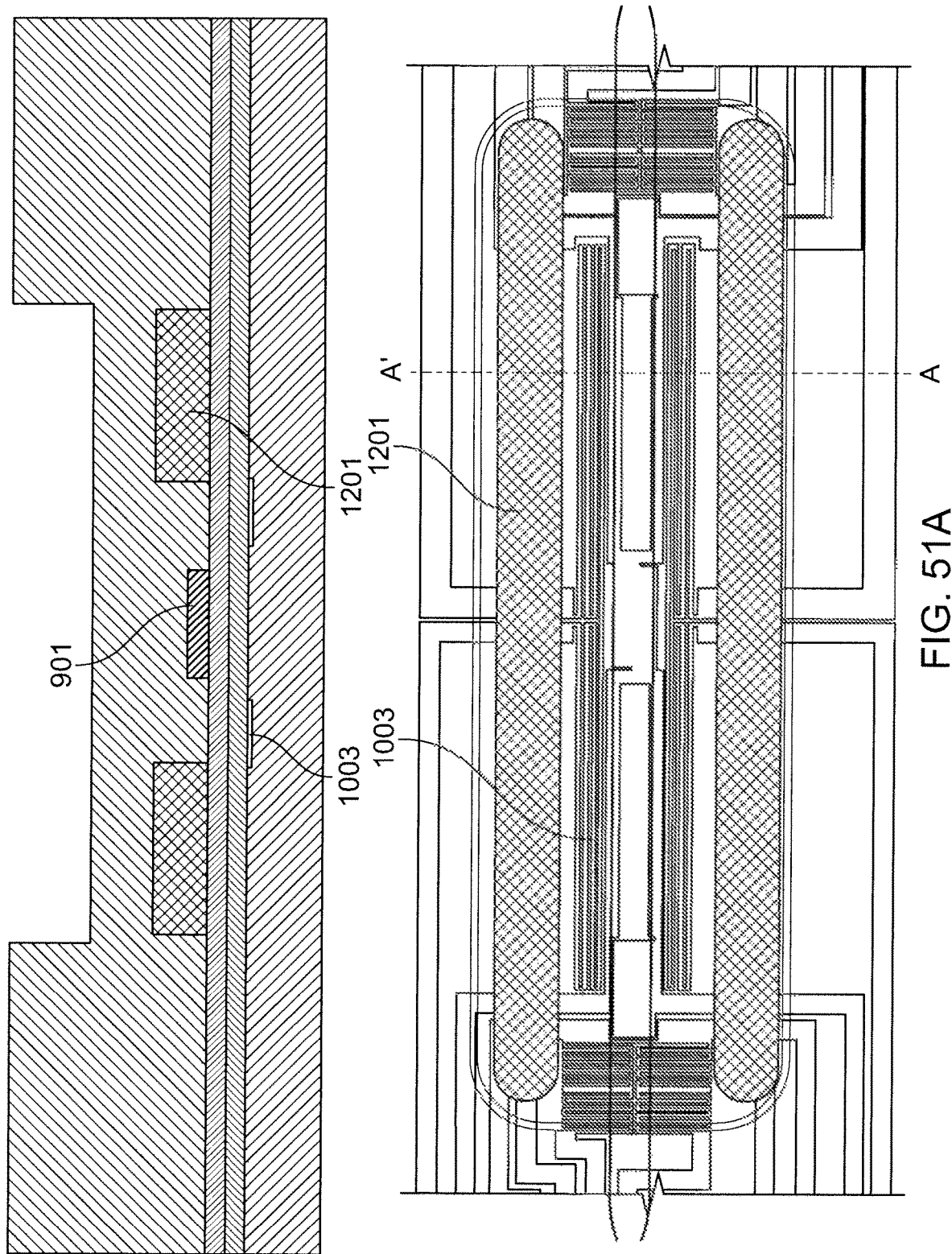


FIG. 50



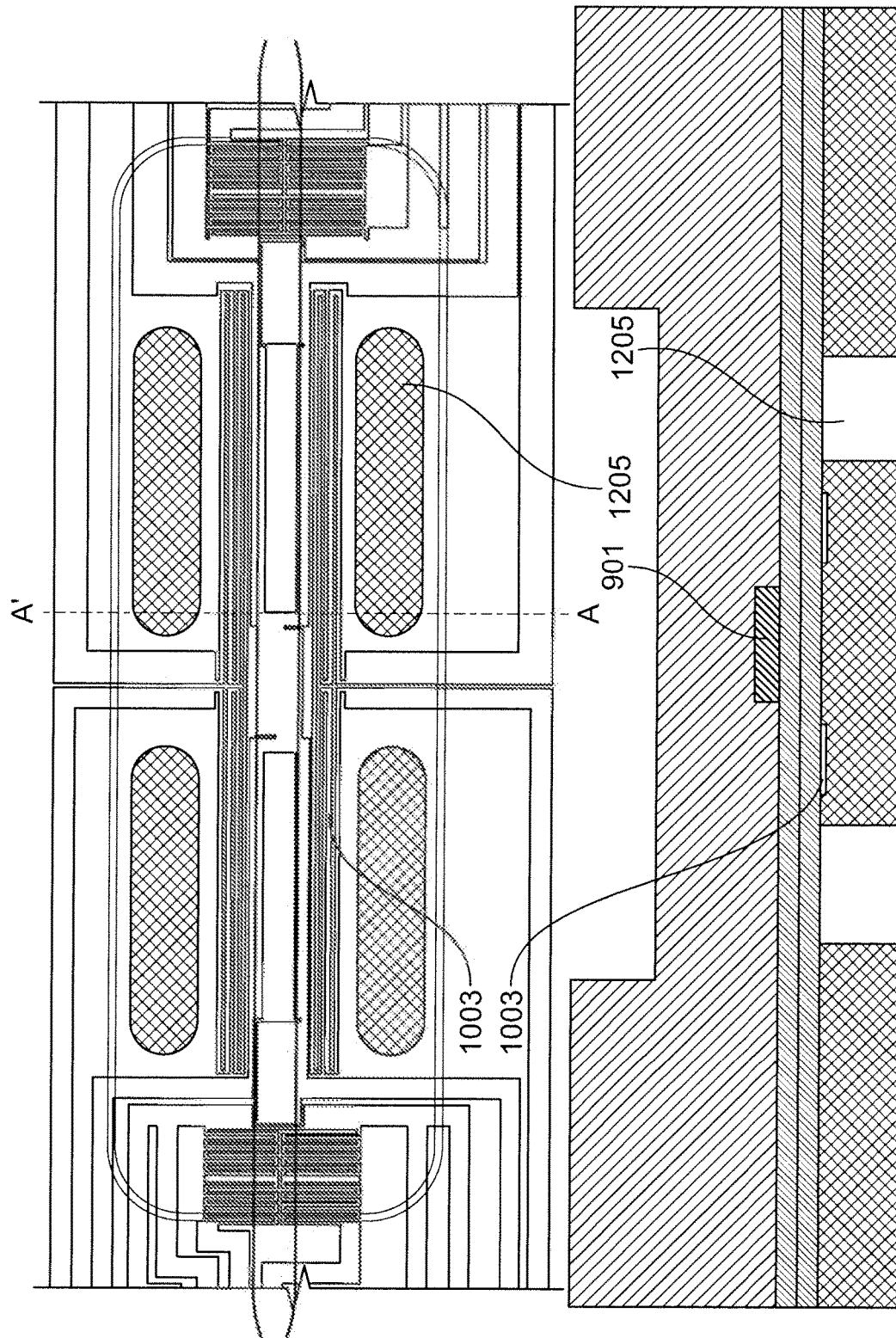


FIG. 51B

U.S. Patent

Apr. 28, 2020

Sheet 77 of 121

US 10,632,466 B1

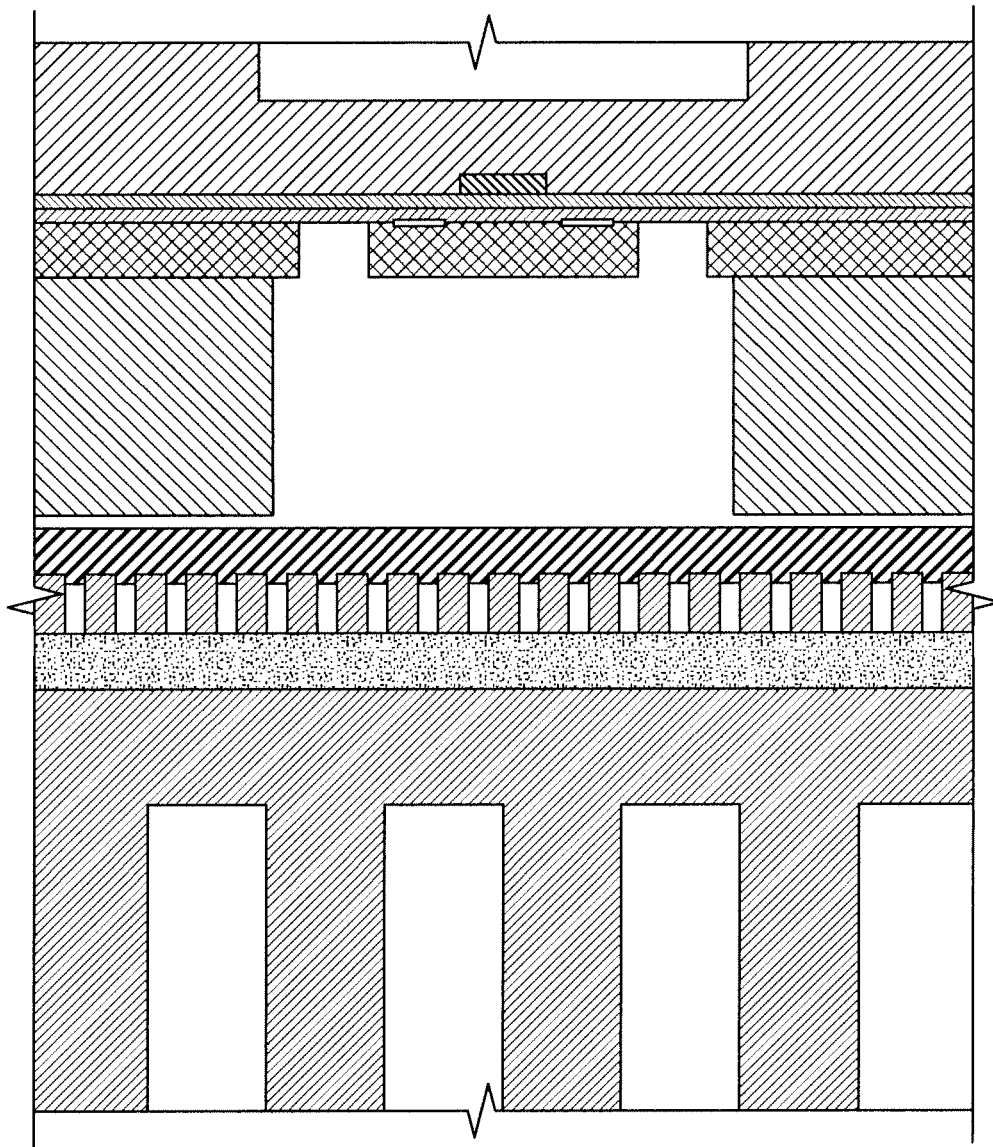


FIG. 51C

U.S. Patent

Apr. 28, 2020

Sheet 78 of 121

US 10,632,466 B1

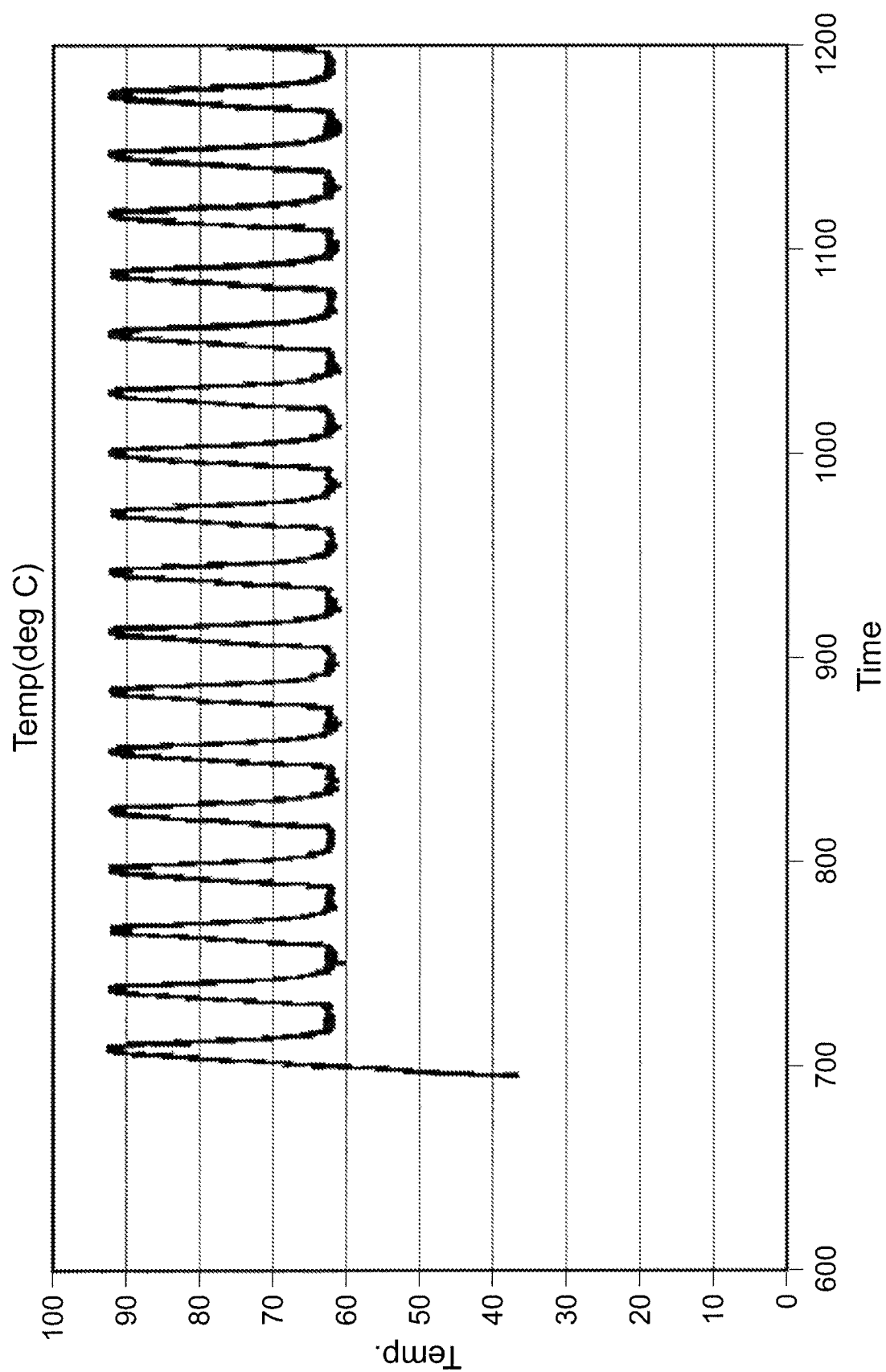


FIG. 52

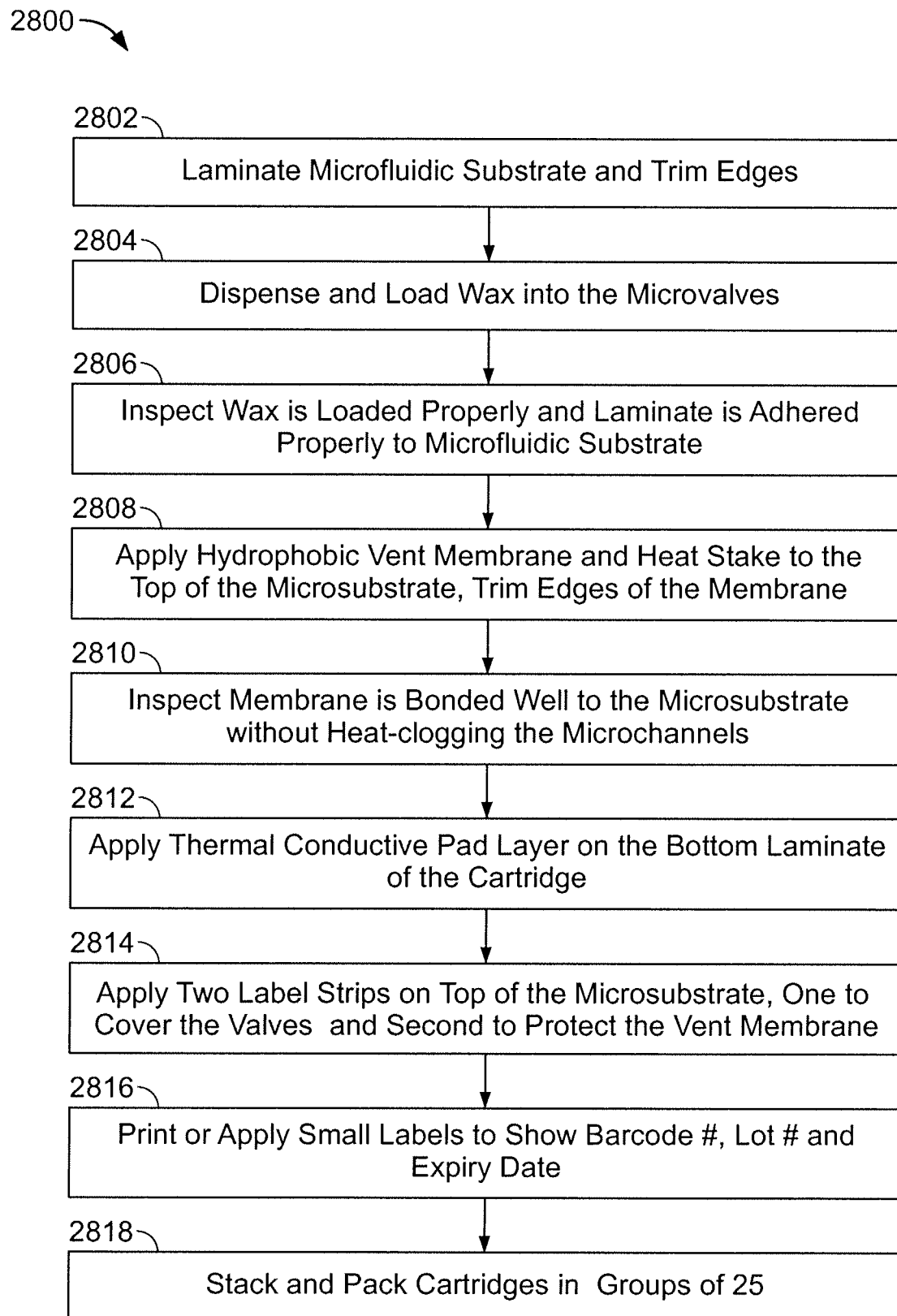


FIG. 53

U.S. Patent

Apr. 28, 2020

Sheet 80 of 121

US 10,632,466 B1

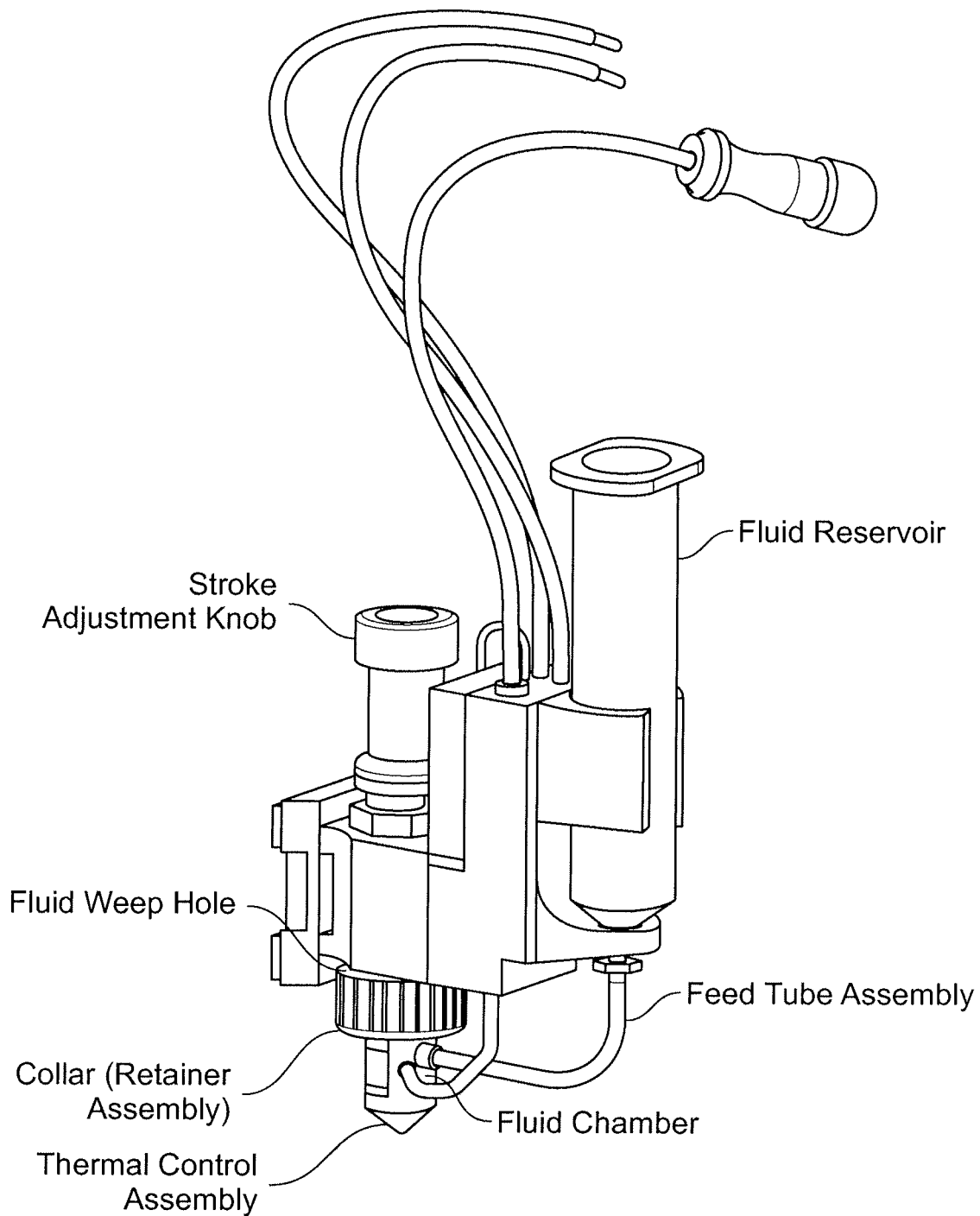


FIG. 54A

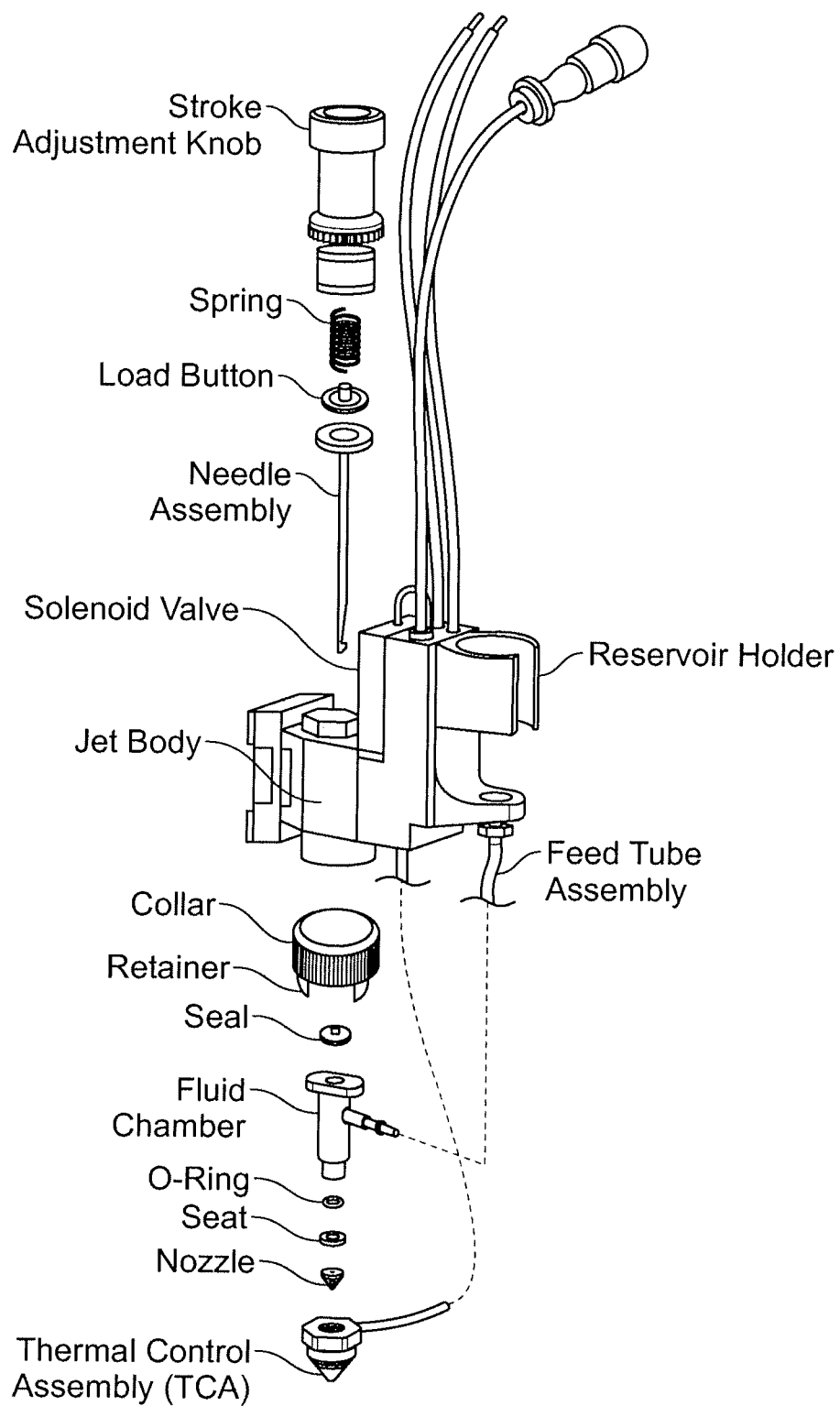


FIG. 54B

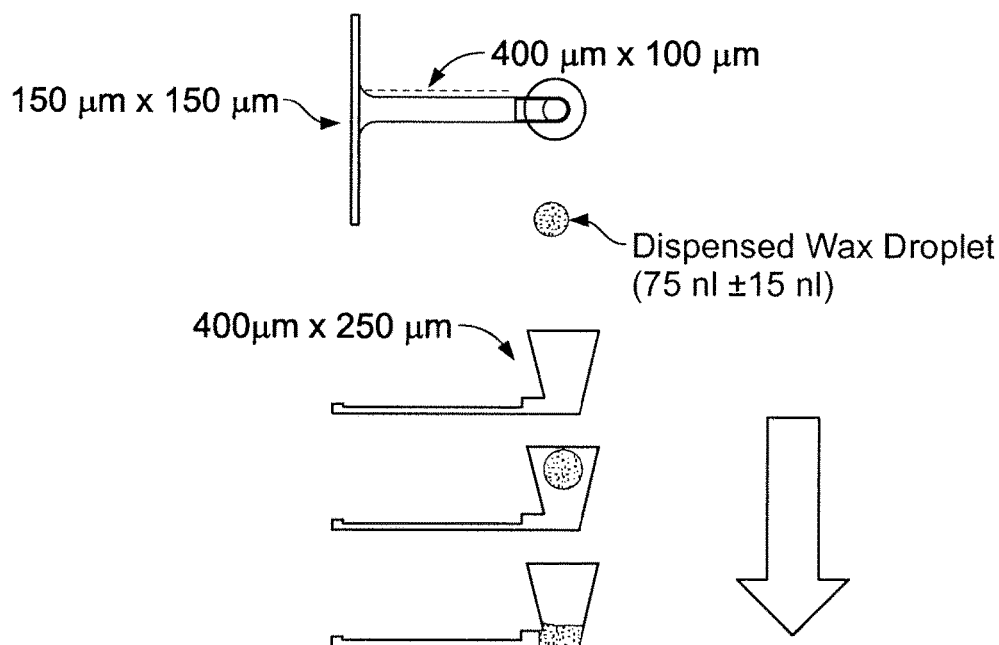


FIG. 55A

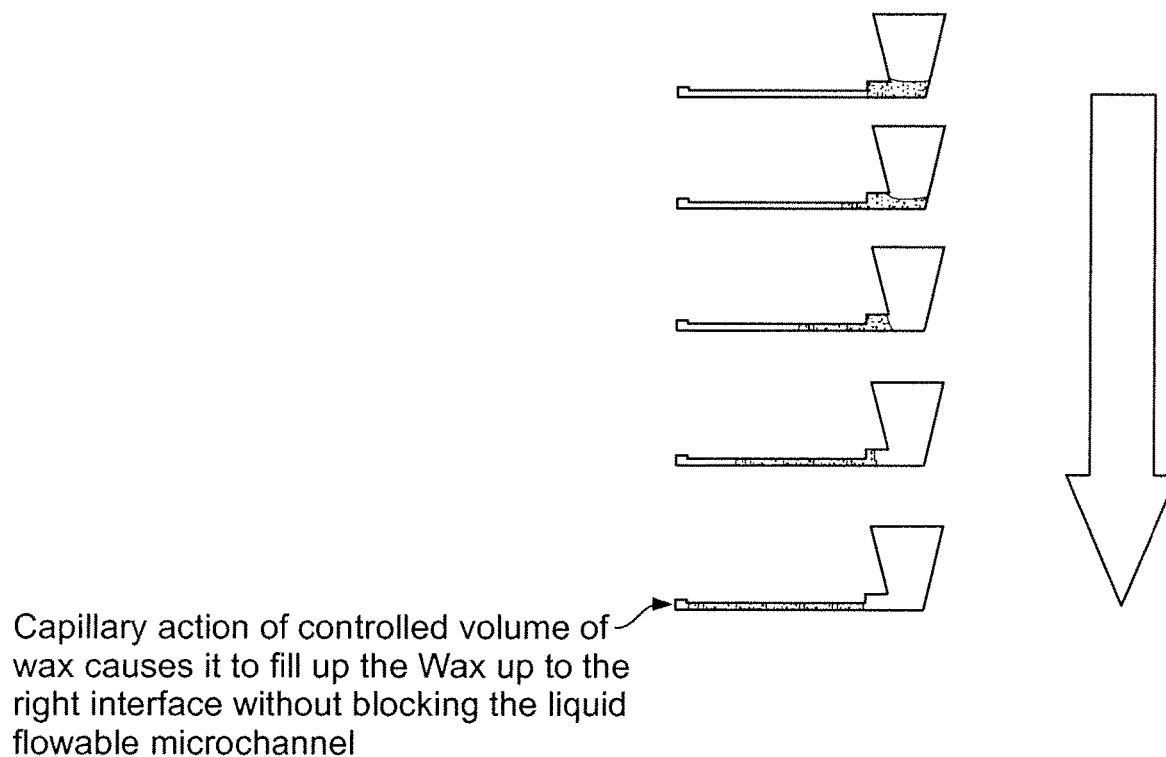


FIG. 55B

U.S. Patent

Apr. 28, 2020

Sheet 83 of 121

US 10,632,466 B1

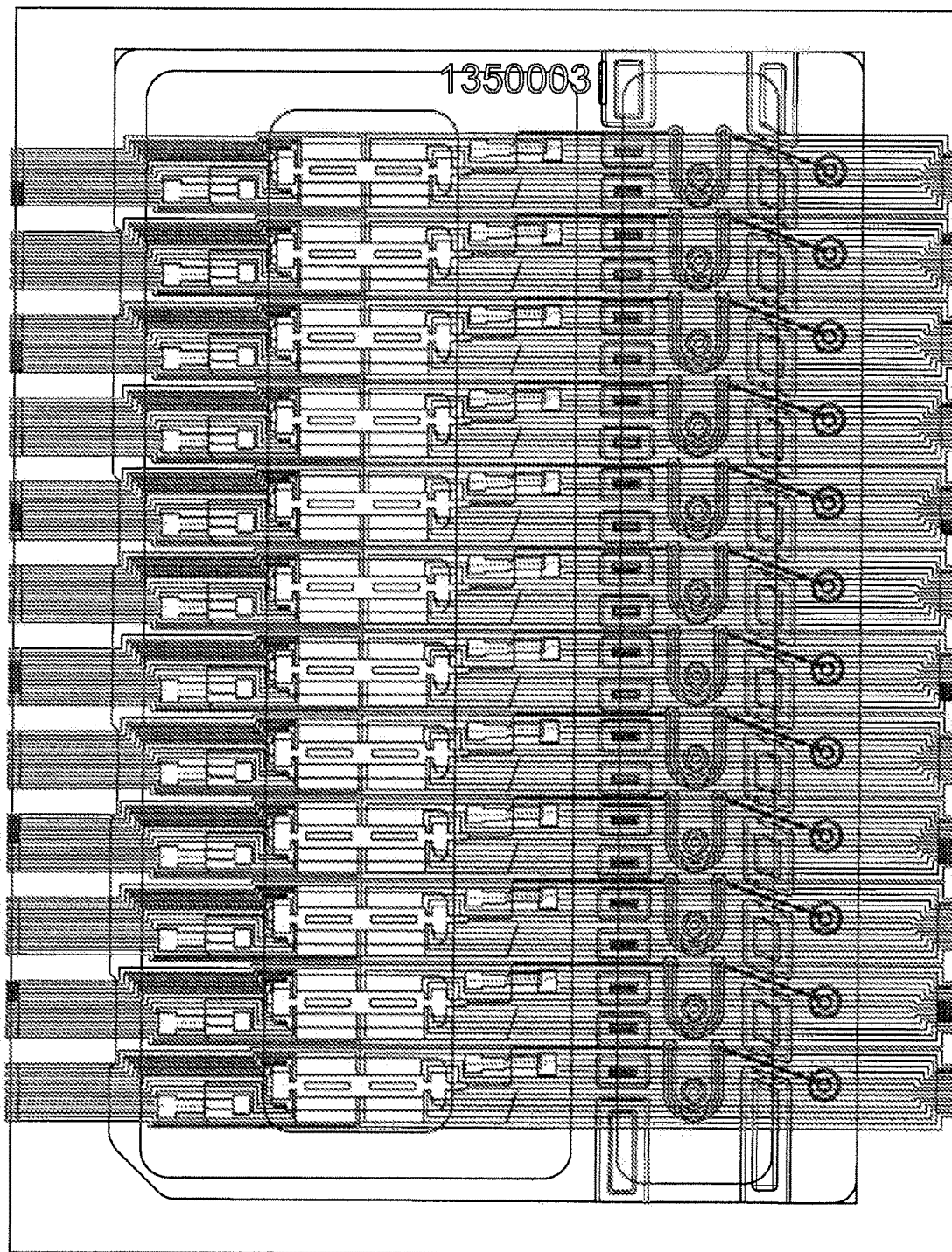


FIG. 56

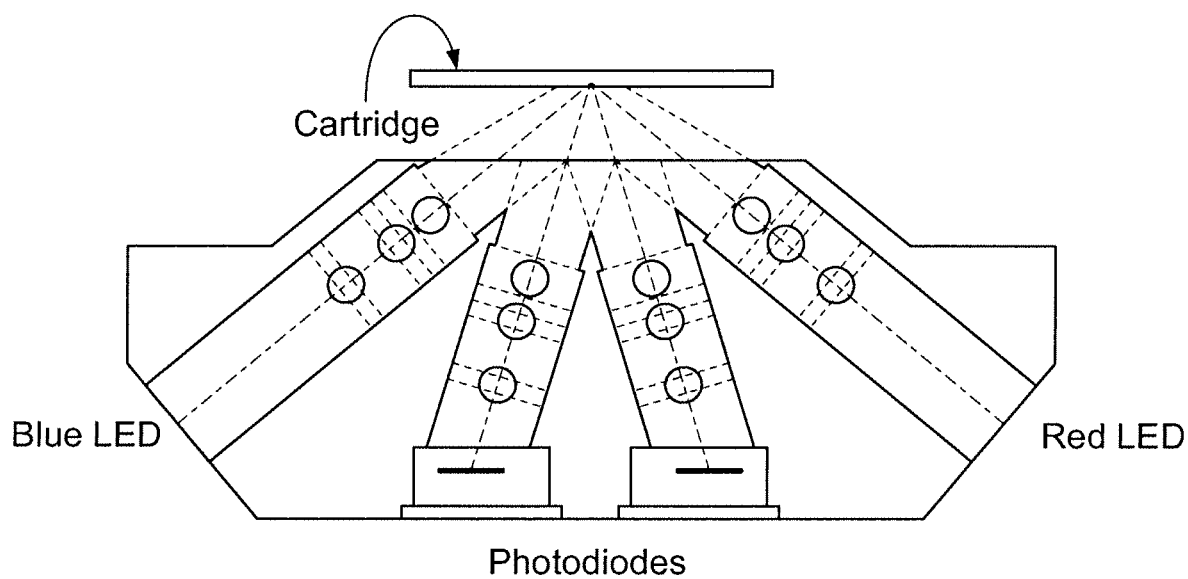


FIG. 57

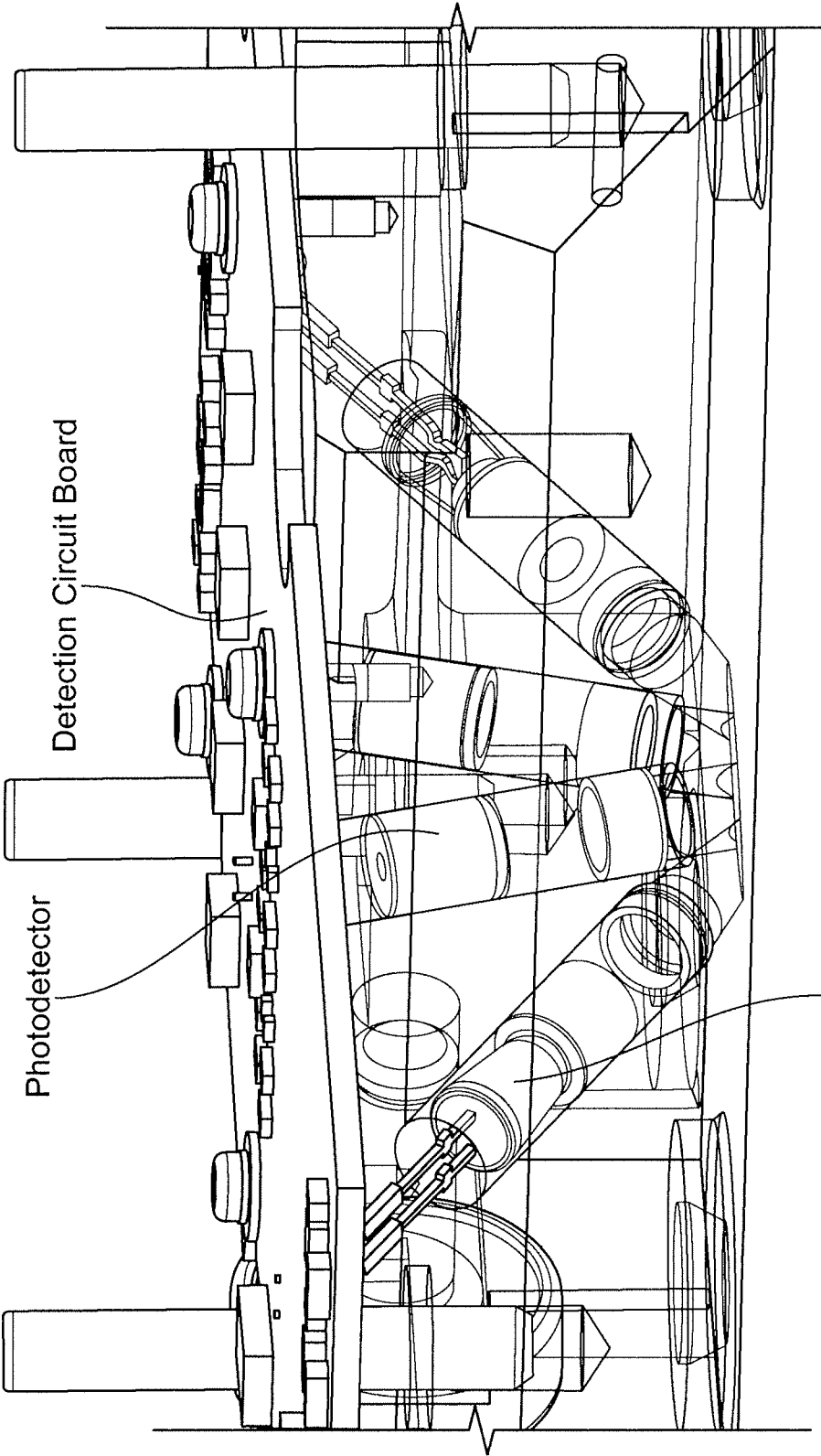


FIG. 58

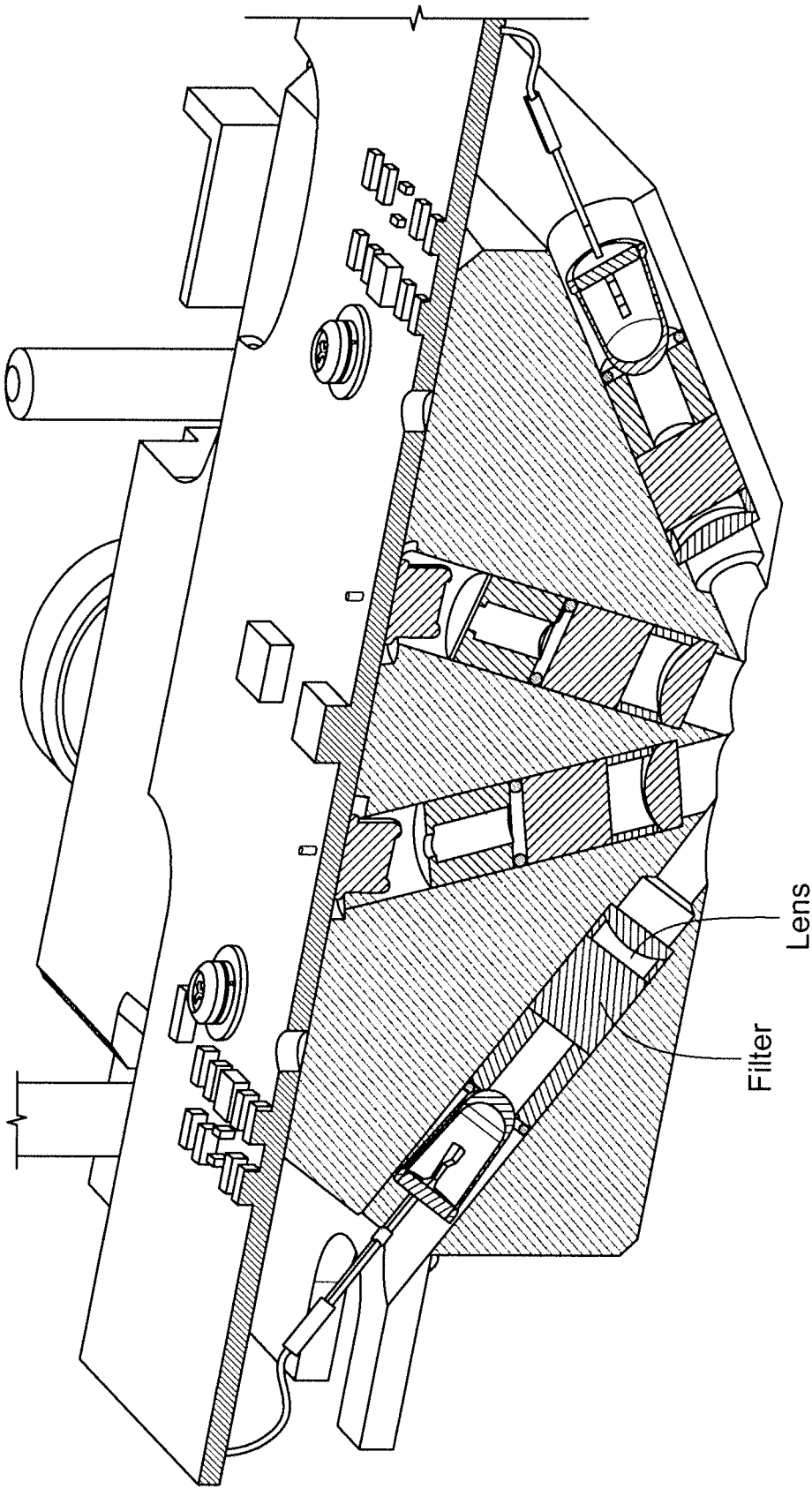


FIG. 59

U.S. Patent

Apr. 28, 2020

Sheet 87 of 121

US 10,632,466 B1

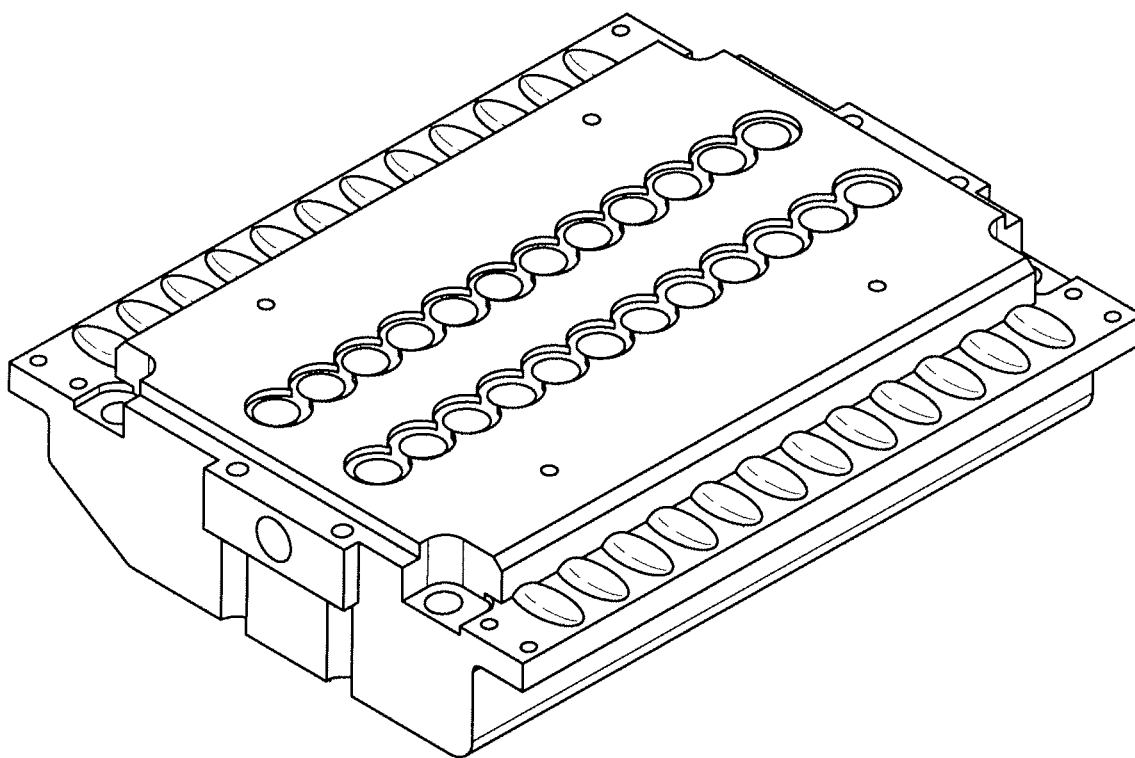


FIG. 60

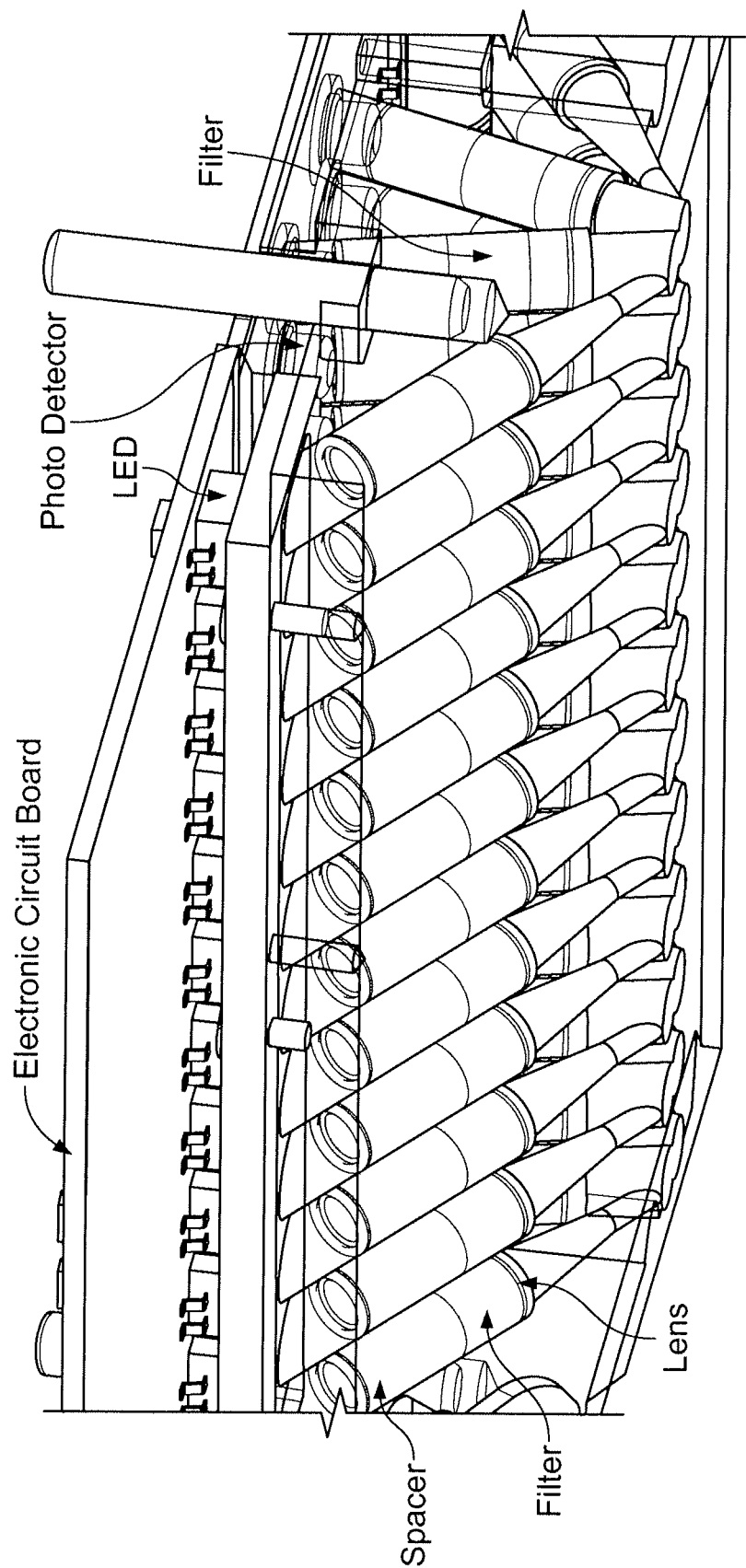


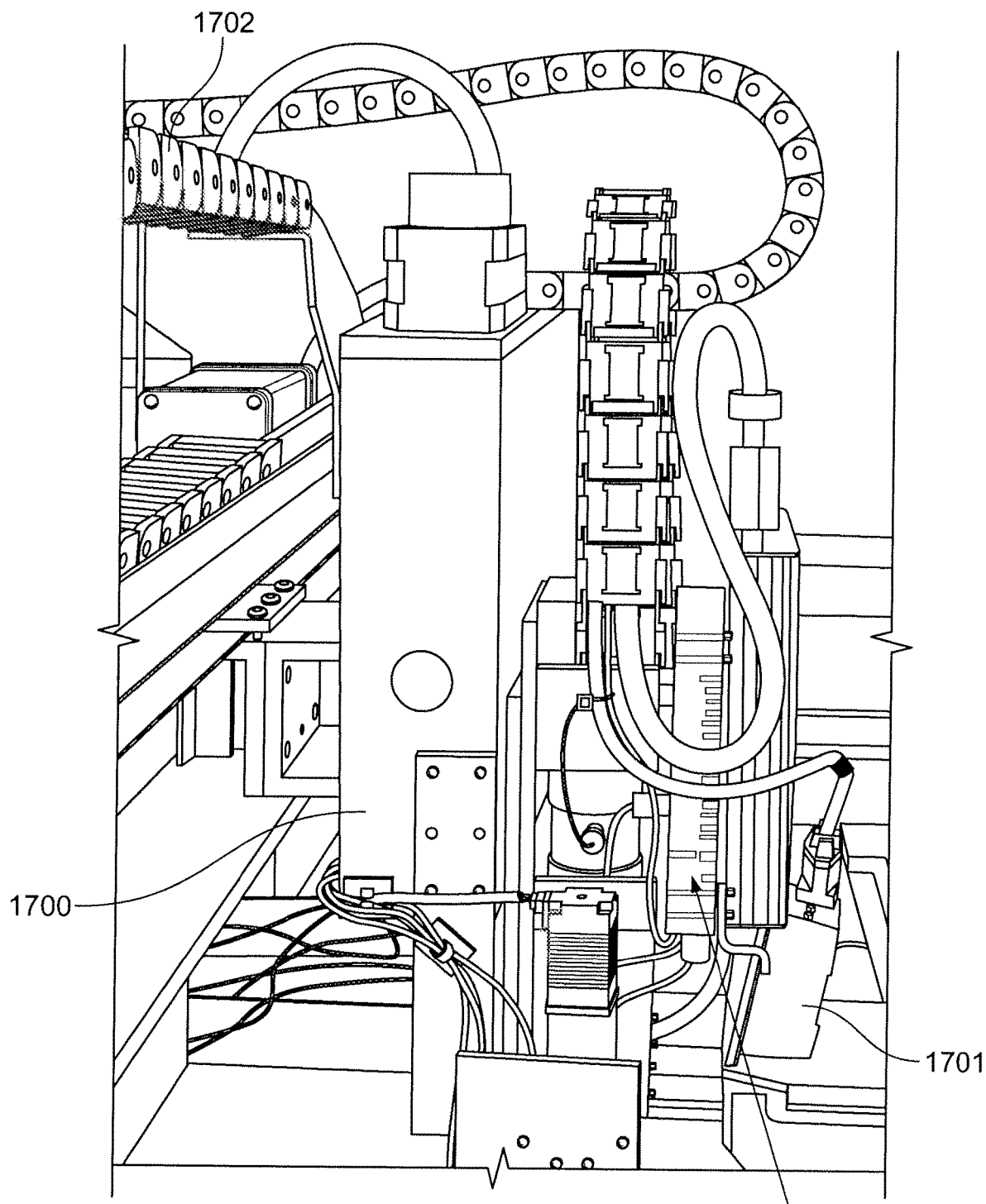
FIG. 61

U.S. Patent

Apr. 28, 2020

Sheet 90 of 121

US 10,632,466 B1



Multi-pipettor Head
Supported and Capable
of Moving Up and Down
in the Z-axis

Fluid Distribution Head
FIG. 63

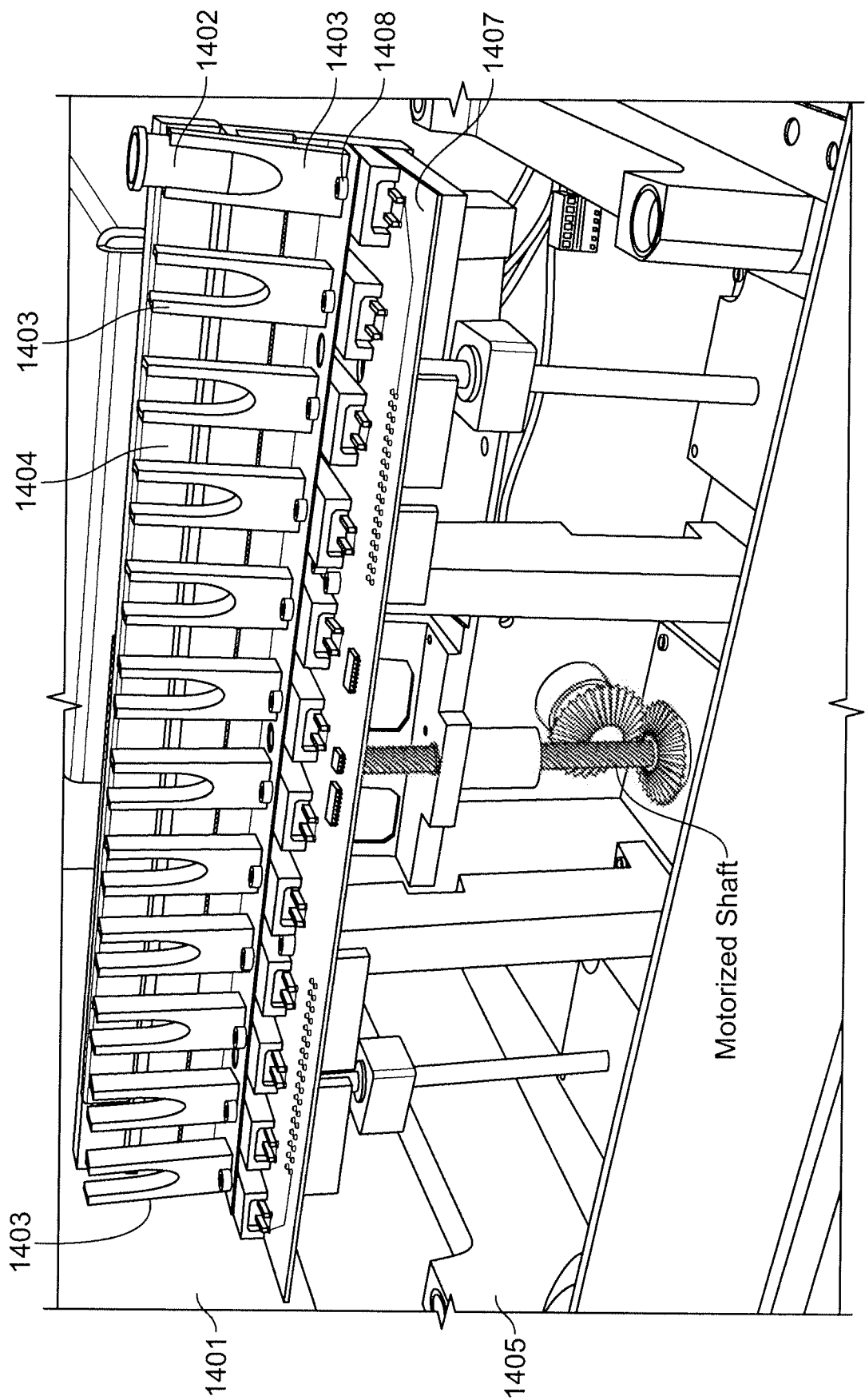


FIG. 64

U.S. Patent

Apr. 28, 2020

Sheet 92 of 121

US 10,632,466 B1

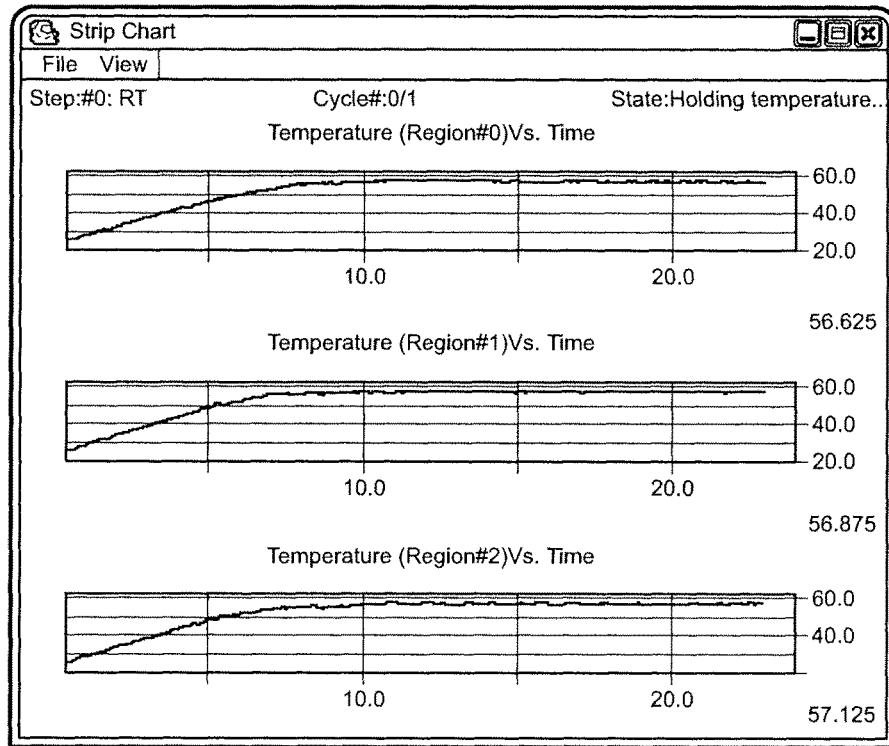


FIG. 65A

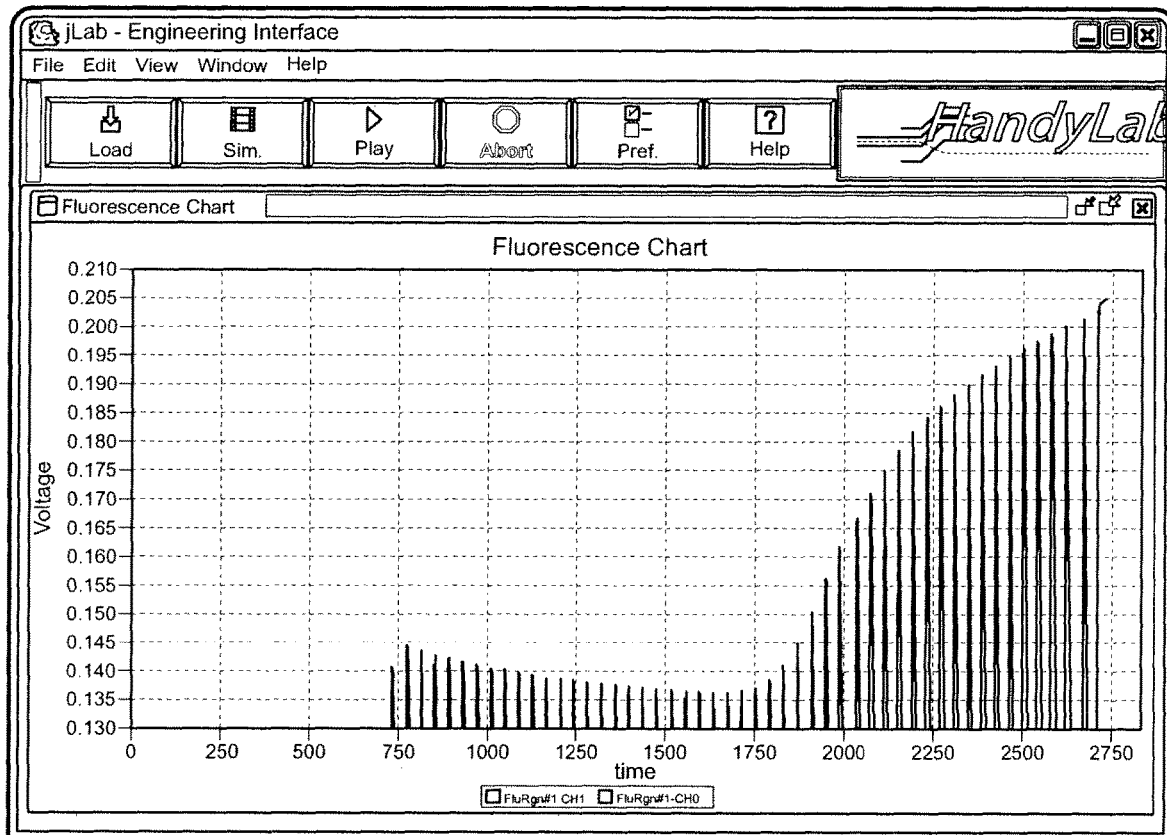


FIG. 65B

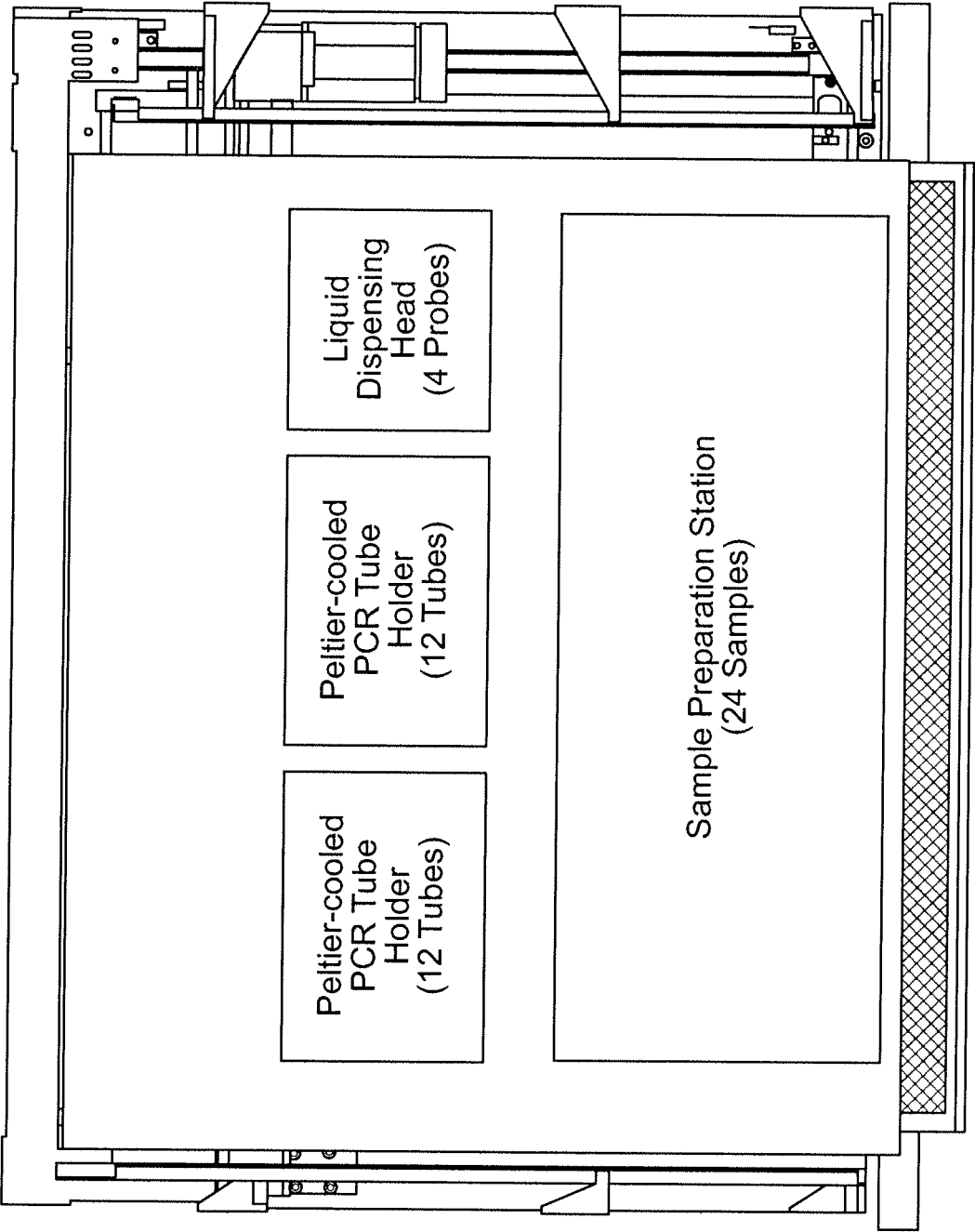
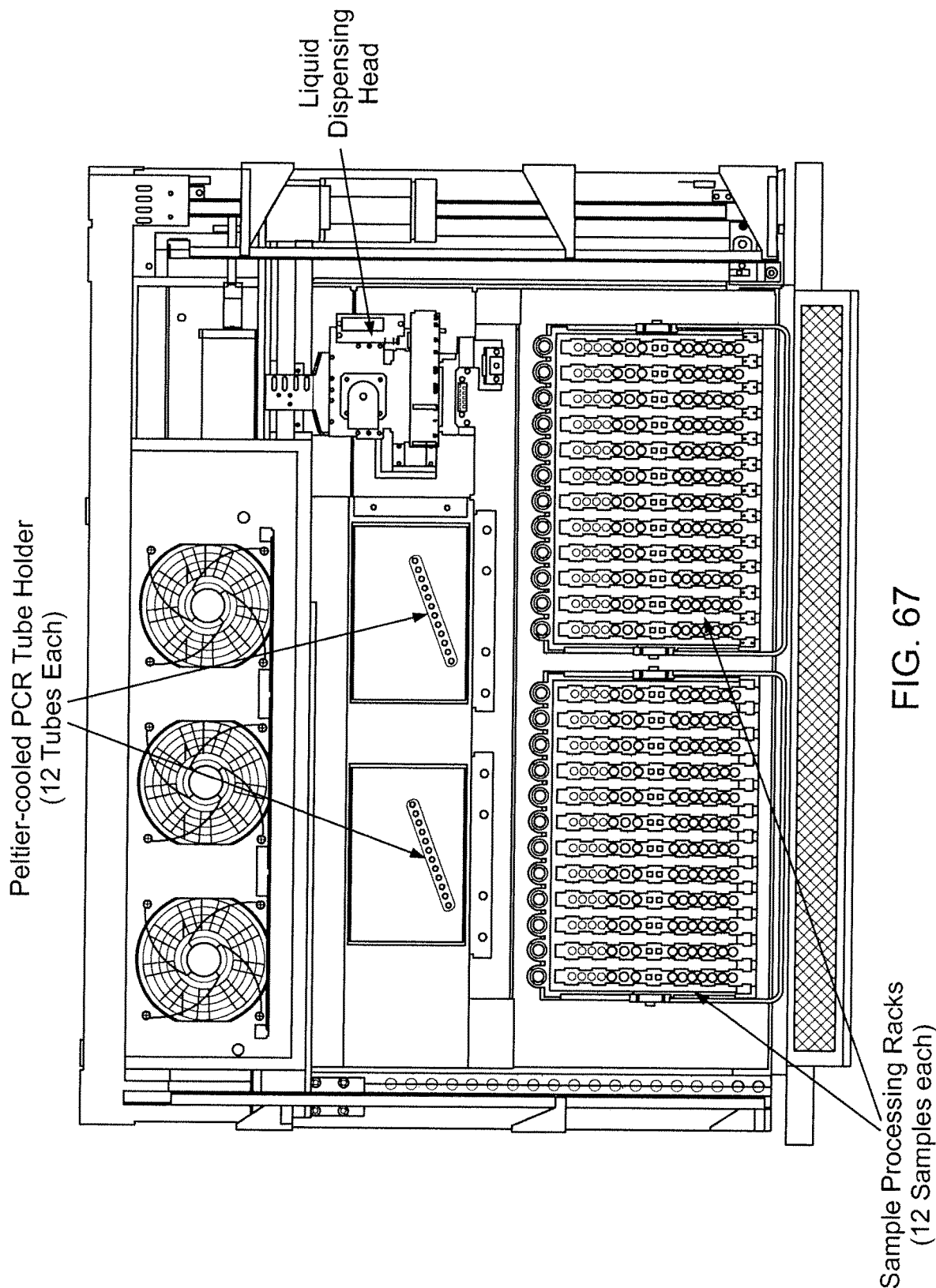
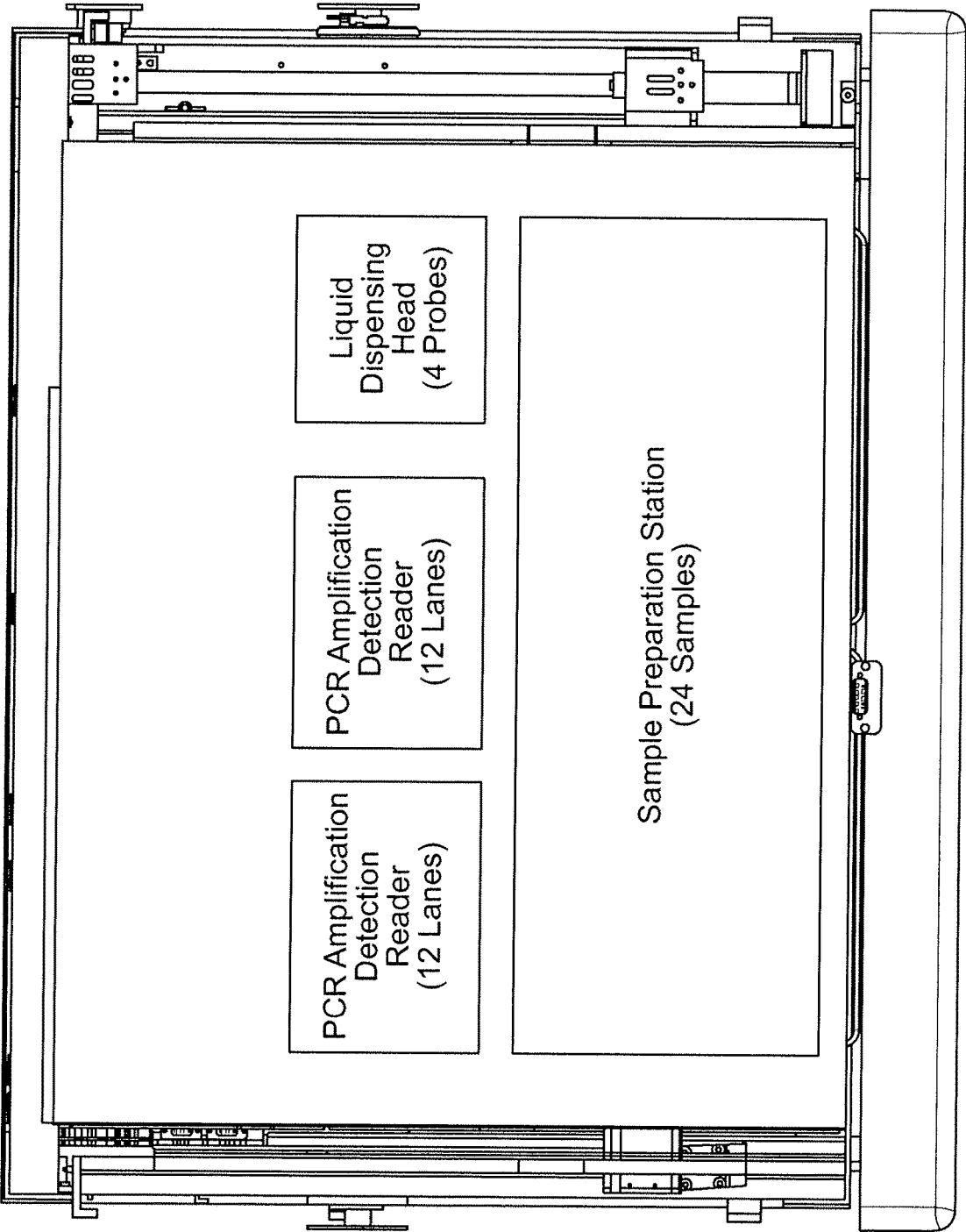


FIG. 66



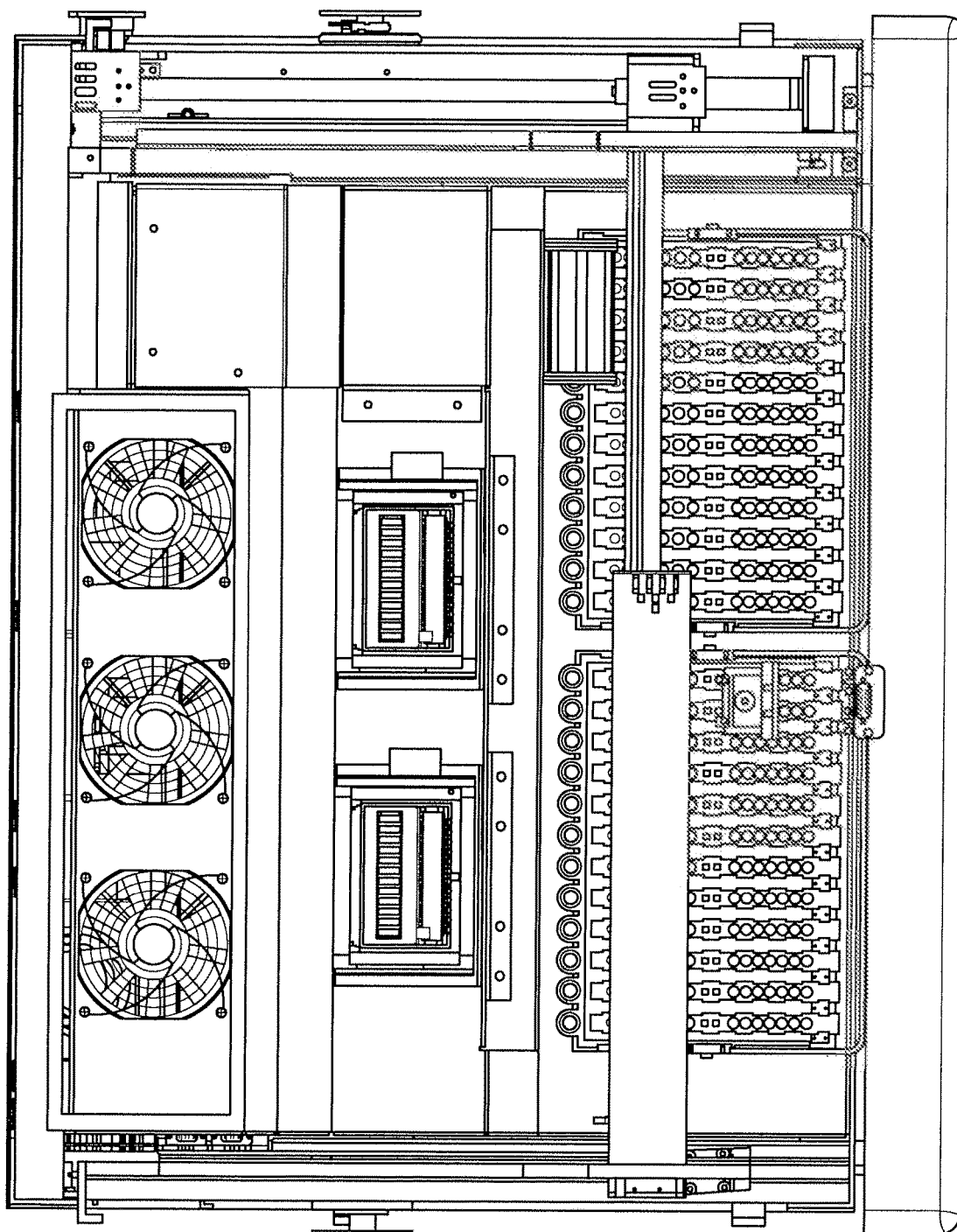


U.S. Patent

Apr. 28, 2020

Sheet 96 of 121

US 10,632,466 B1



U.S. Patent

Apr. 28, 2020

Sheet 97 of 121

US 10,632,466 B1

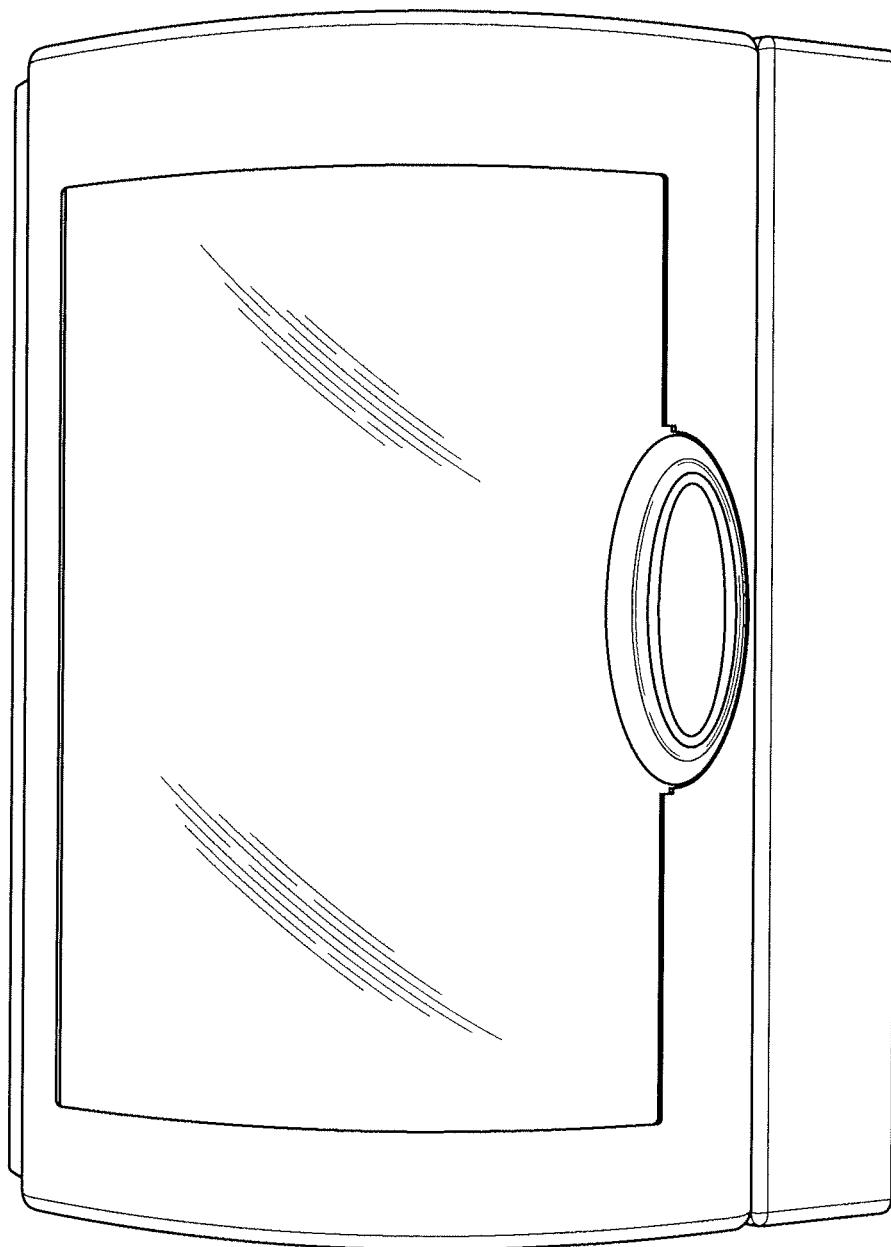


FIG. 70

U.S. Patent

Apr. 28, 2020

Sheet 98 of 121

US 10,632,466 B1

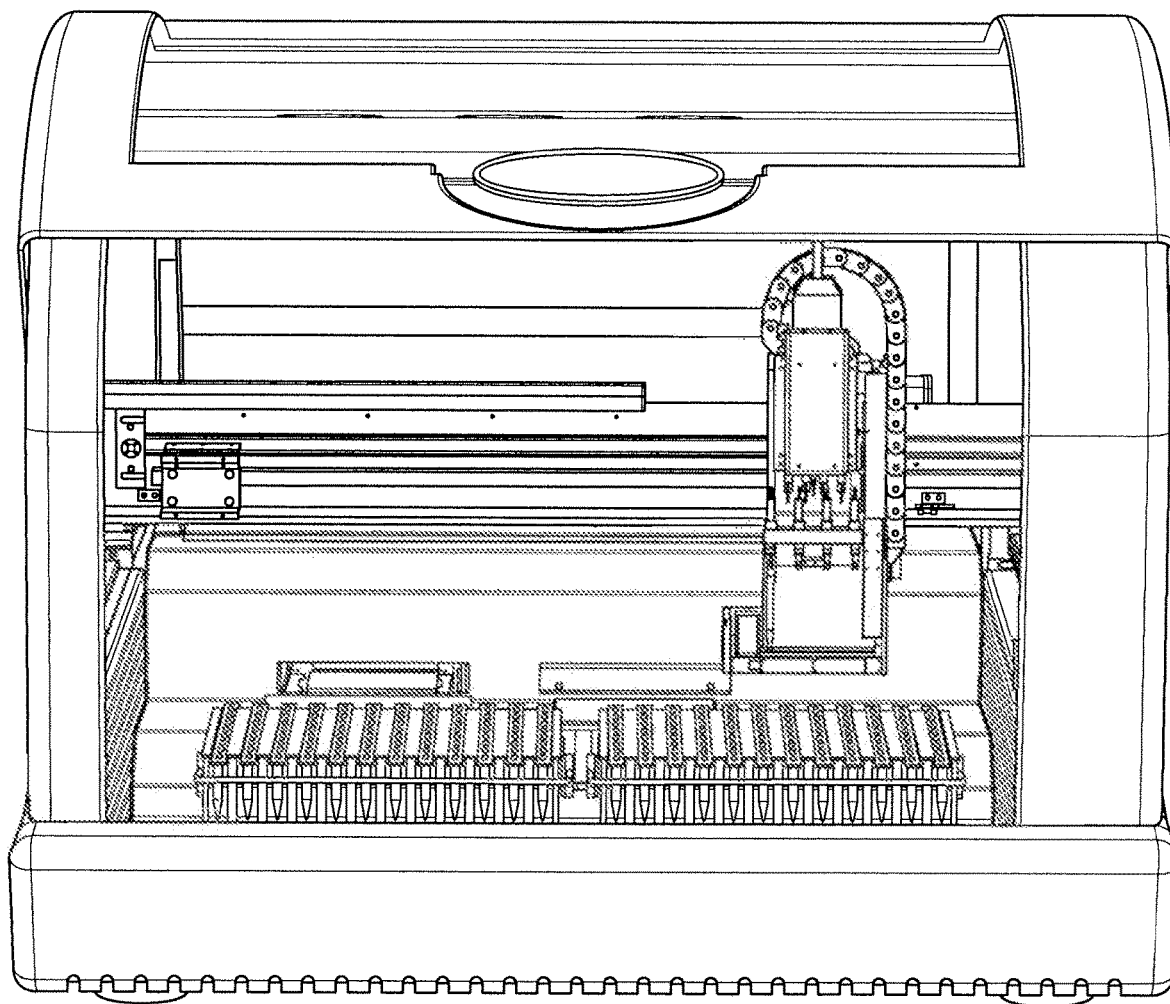
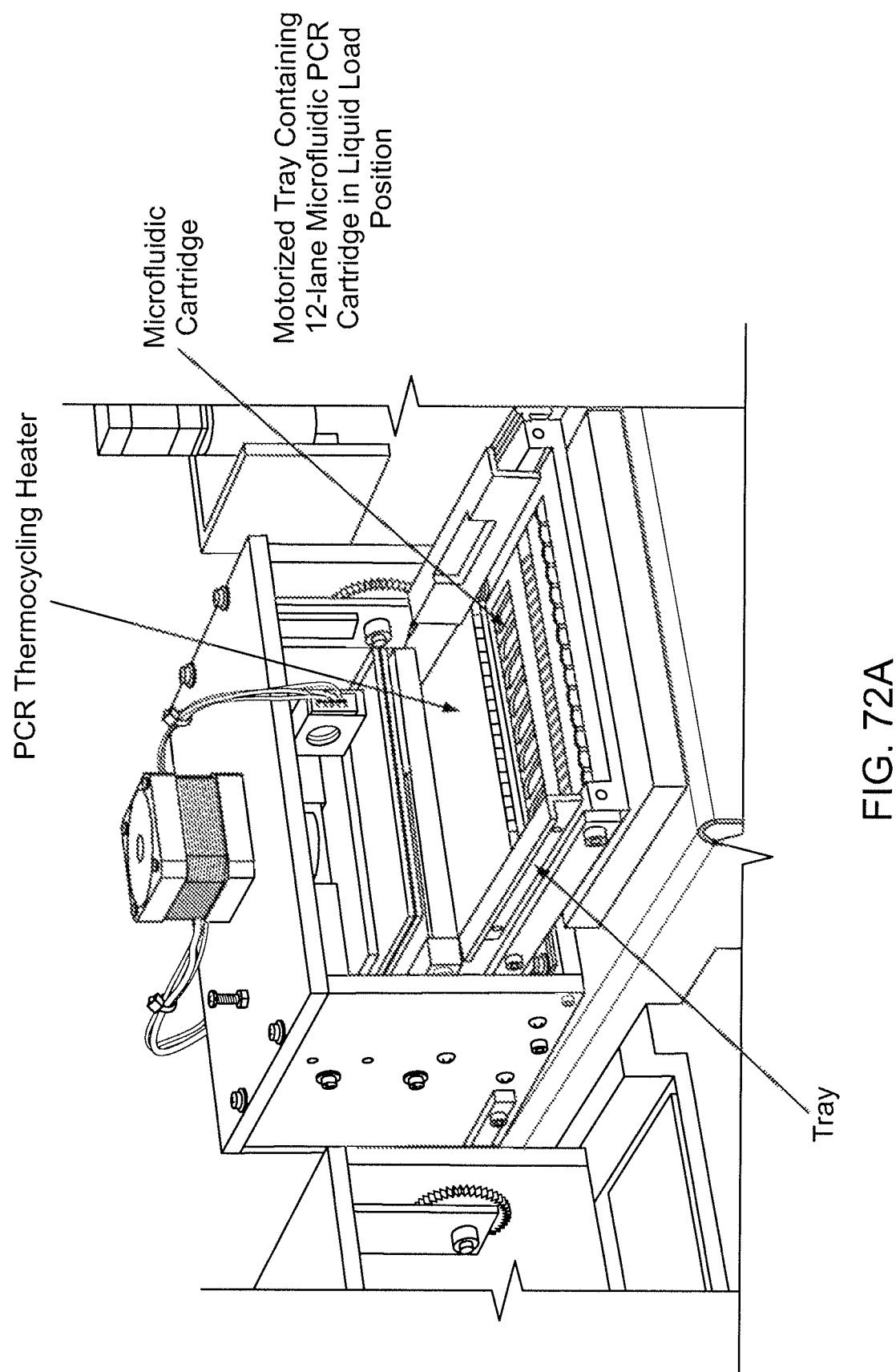
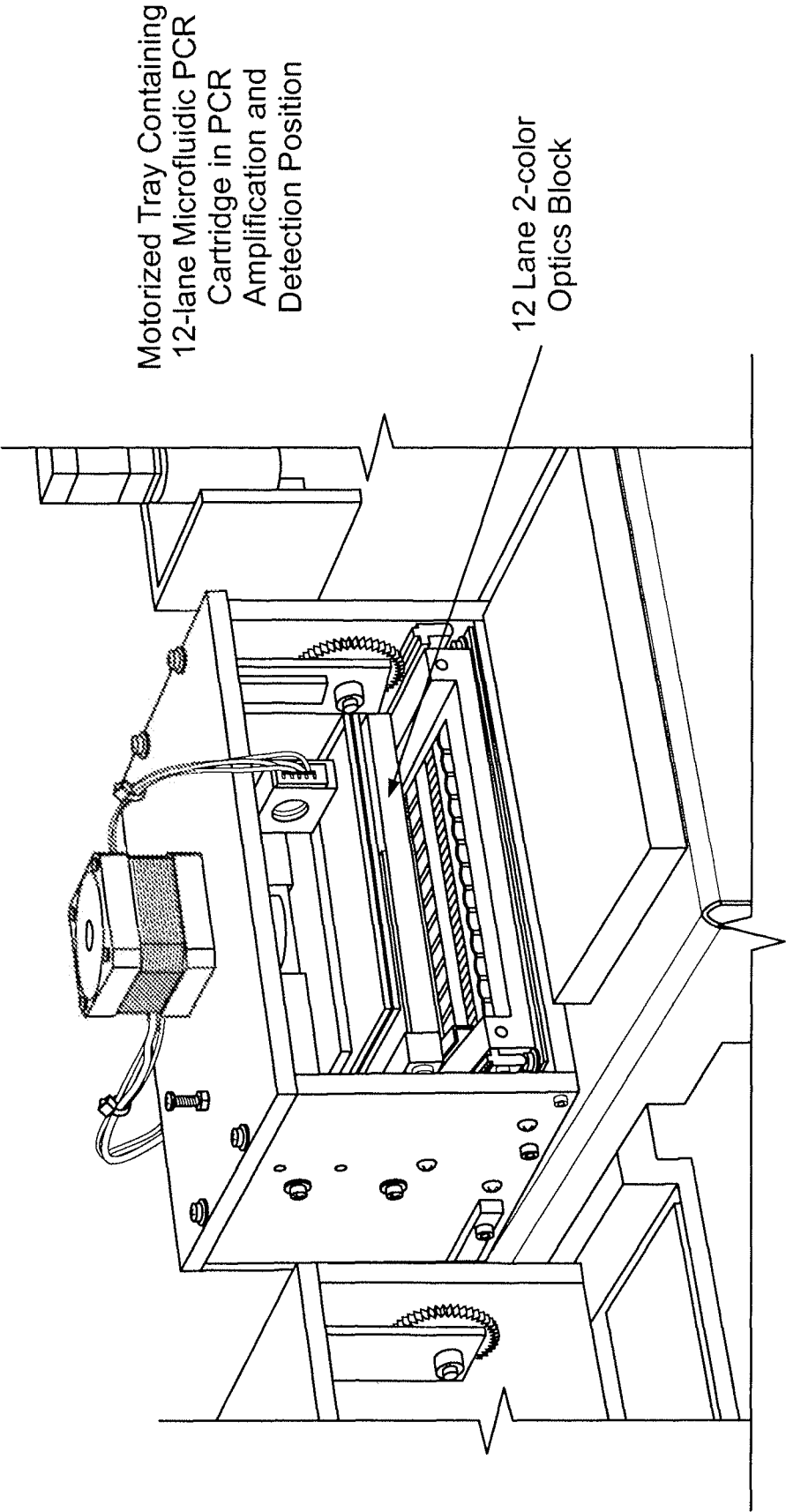


FIG. 71





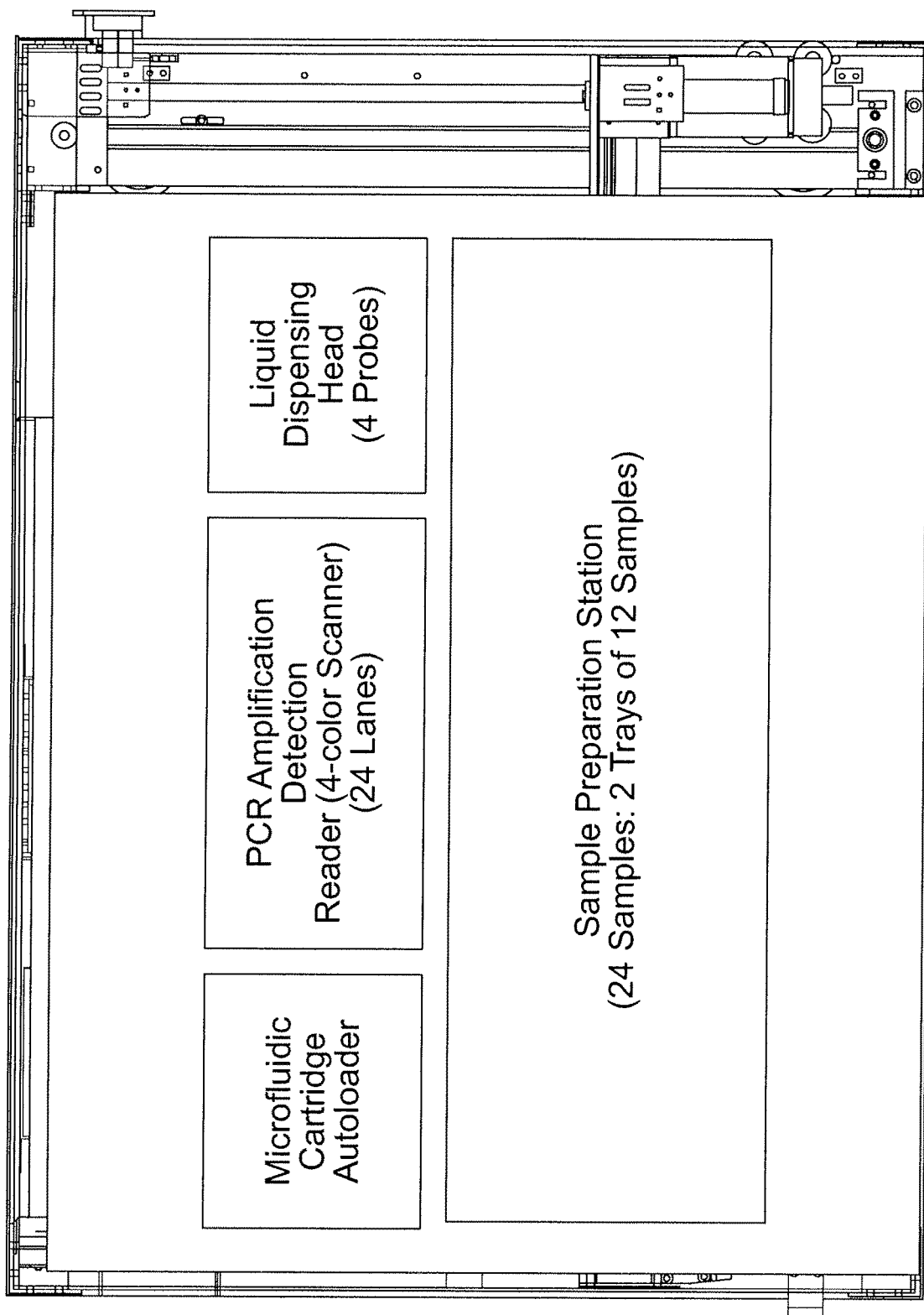


FIG. 73

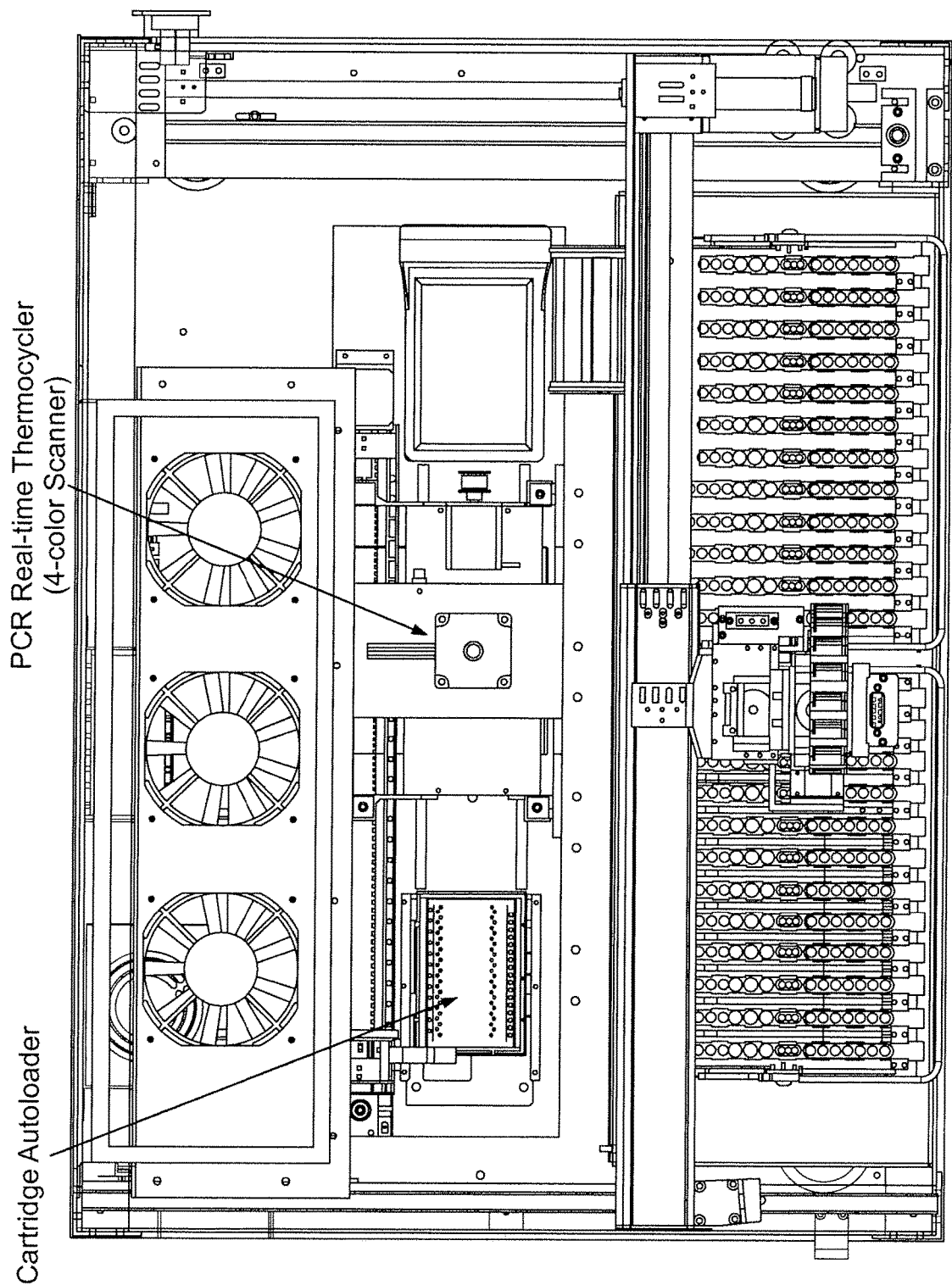


FIG. 74

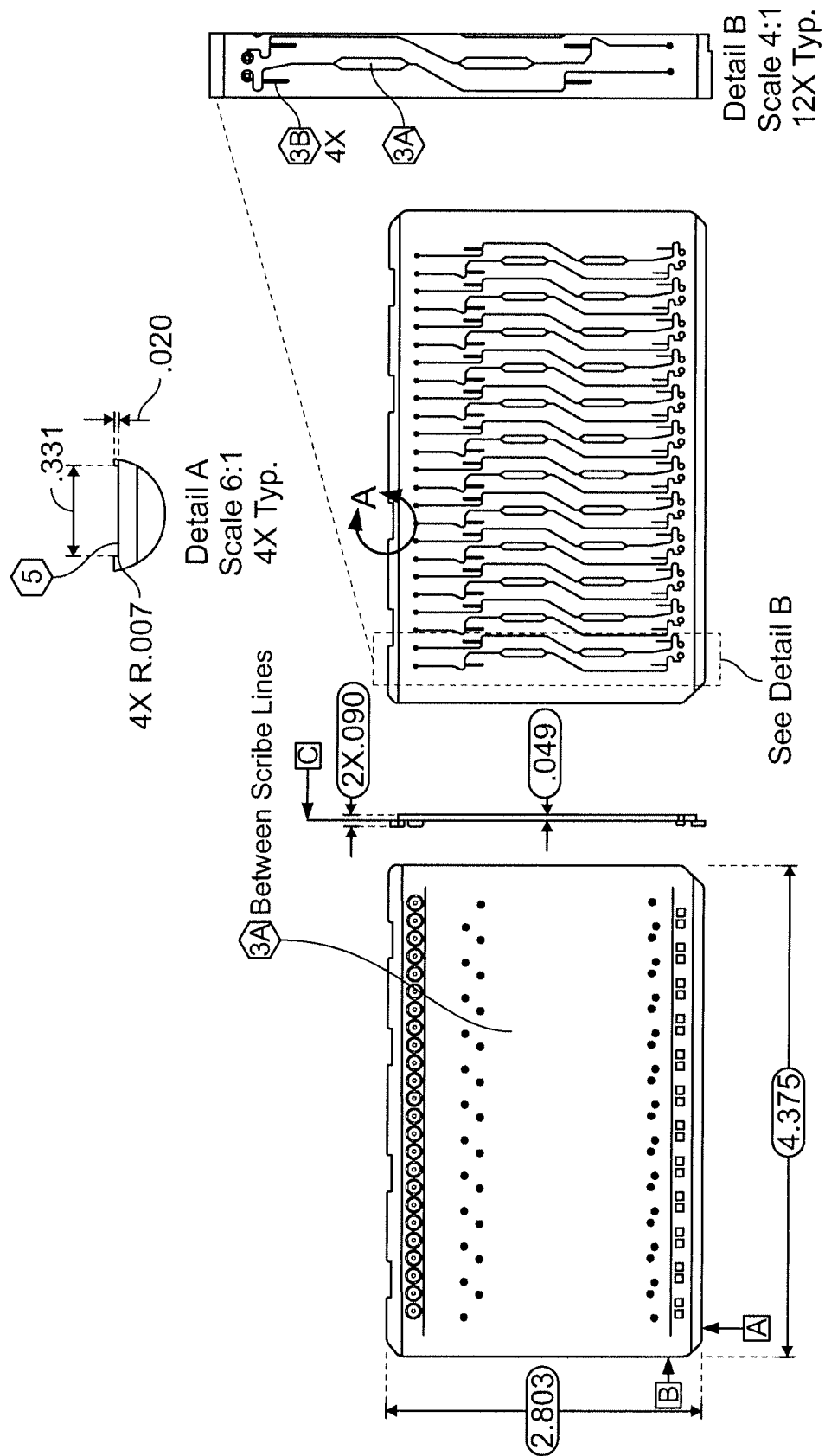


FIG. 75

U.S. Patent

Apr. 28, 2020

Sheet 104 of 121

US 10,632,466 B1

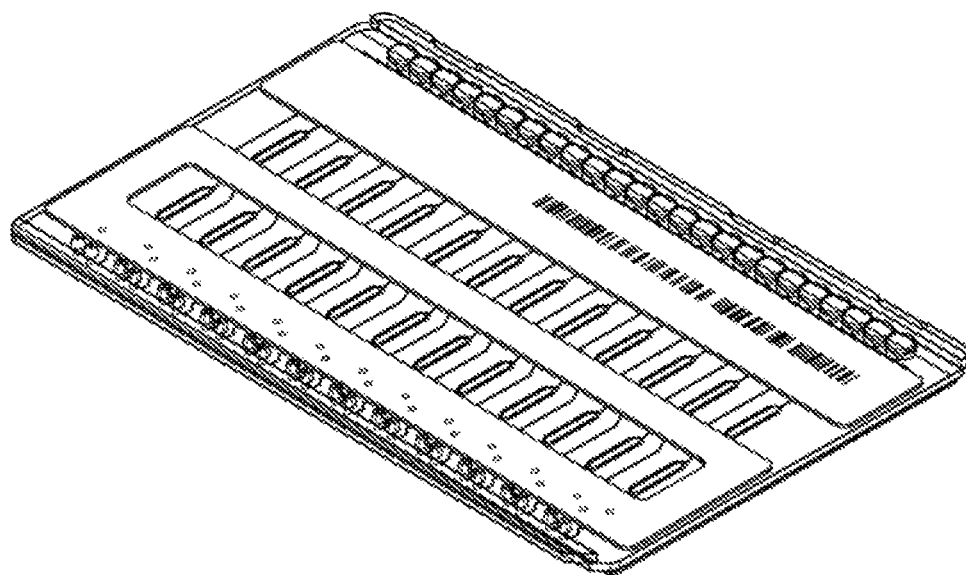


FIG. 76

U.S. Patent

Apr. 28, 2020

Sheet 105 of 121

US 10,632,466 B1

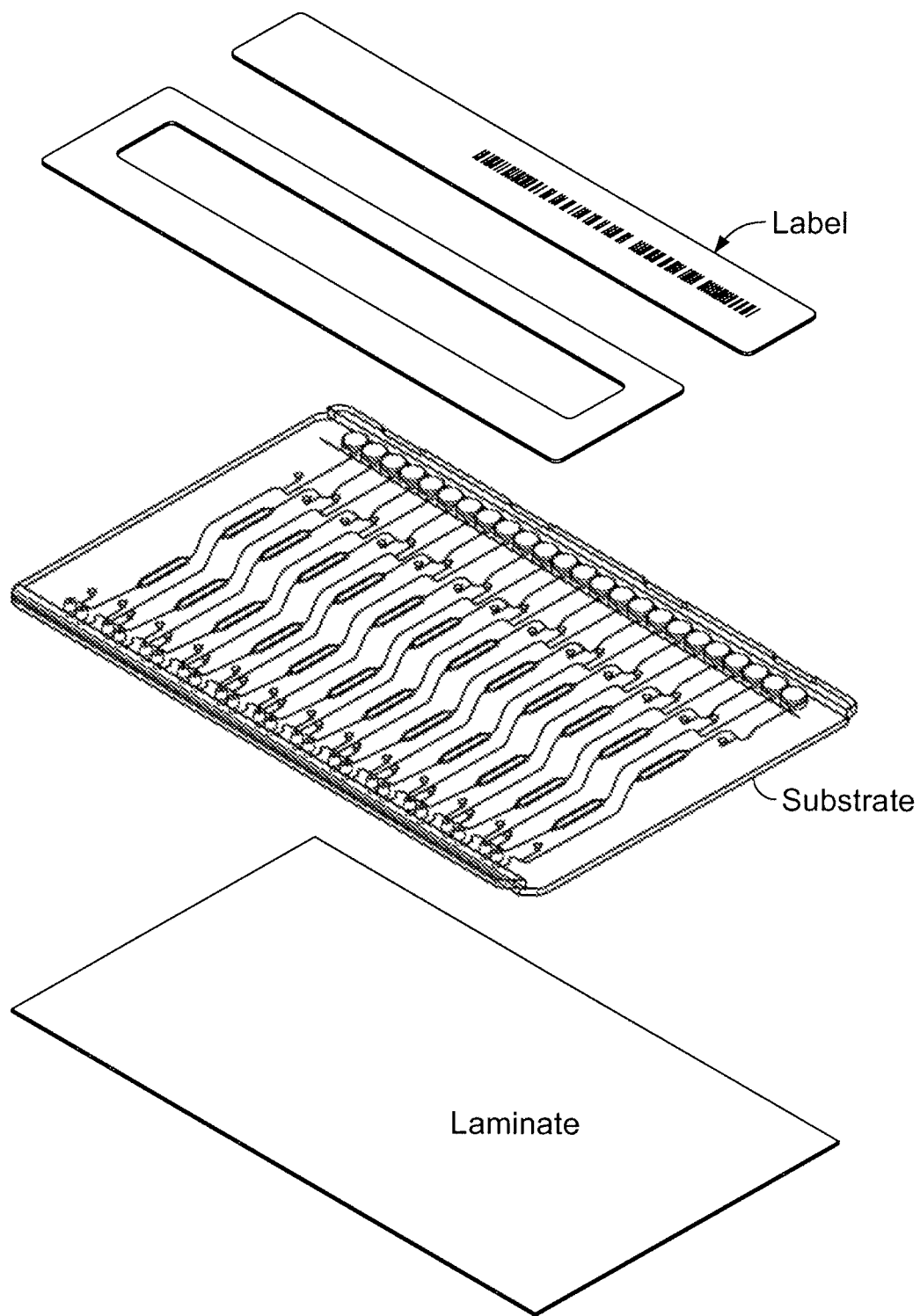
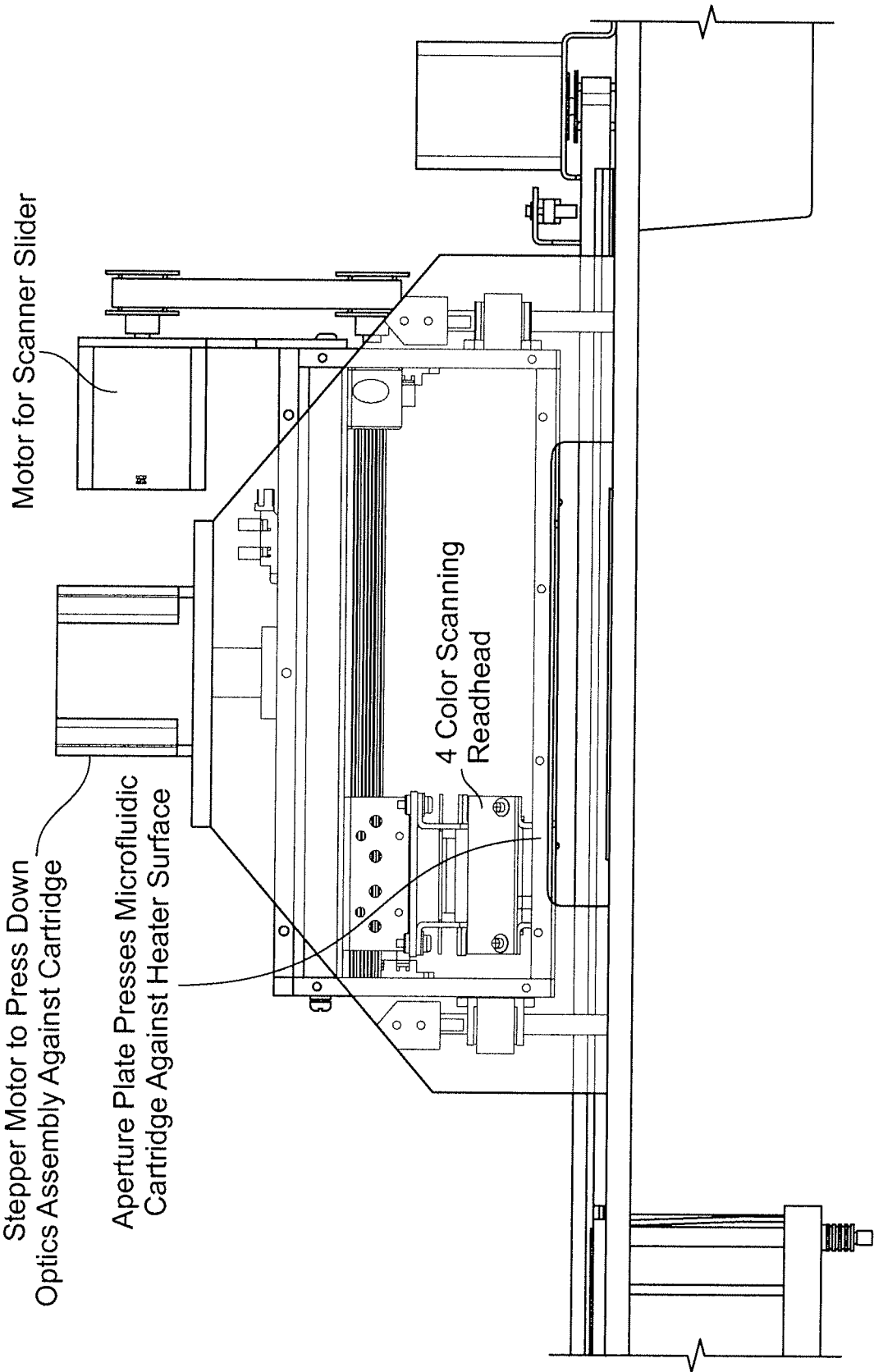


FIG. 77



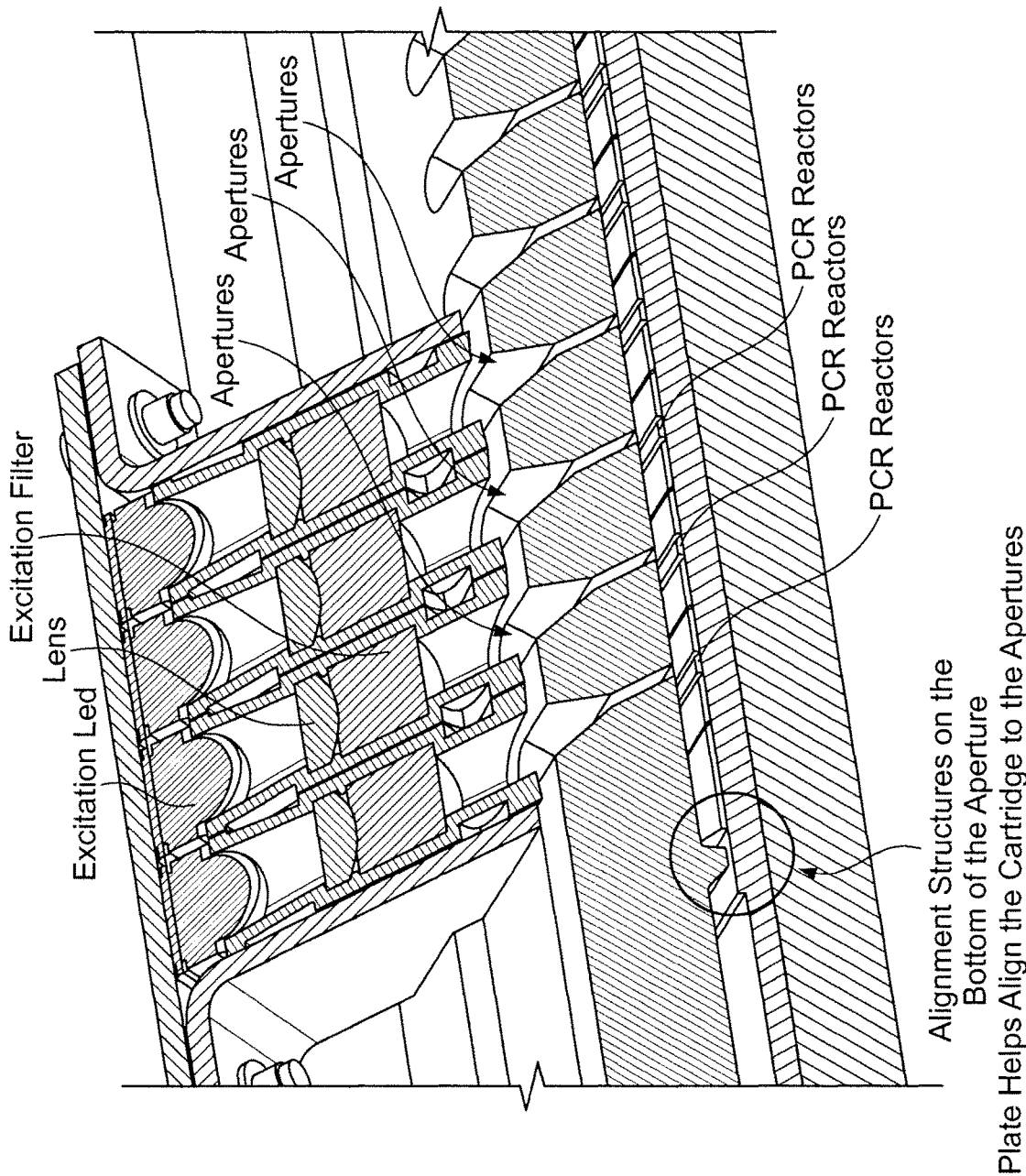
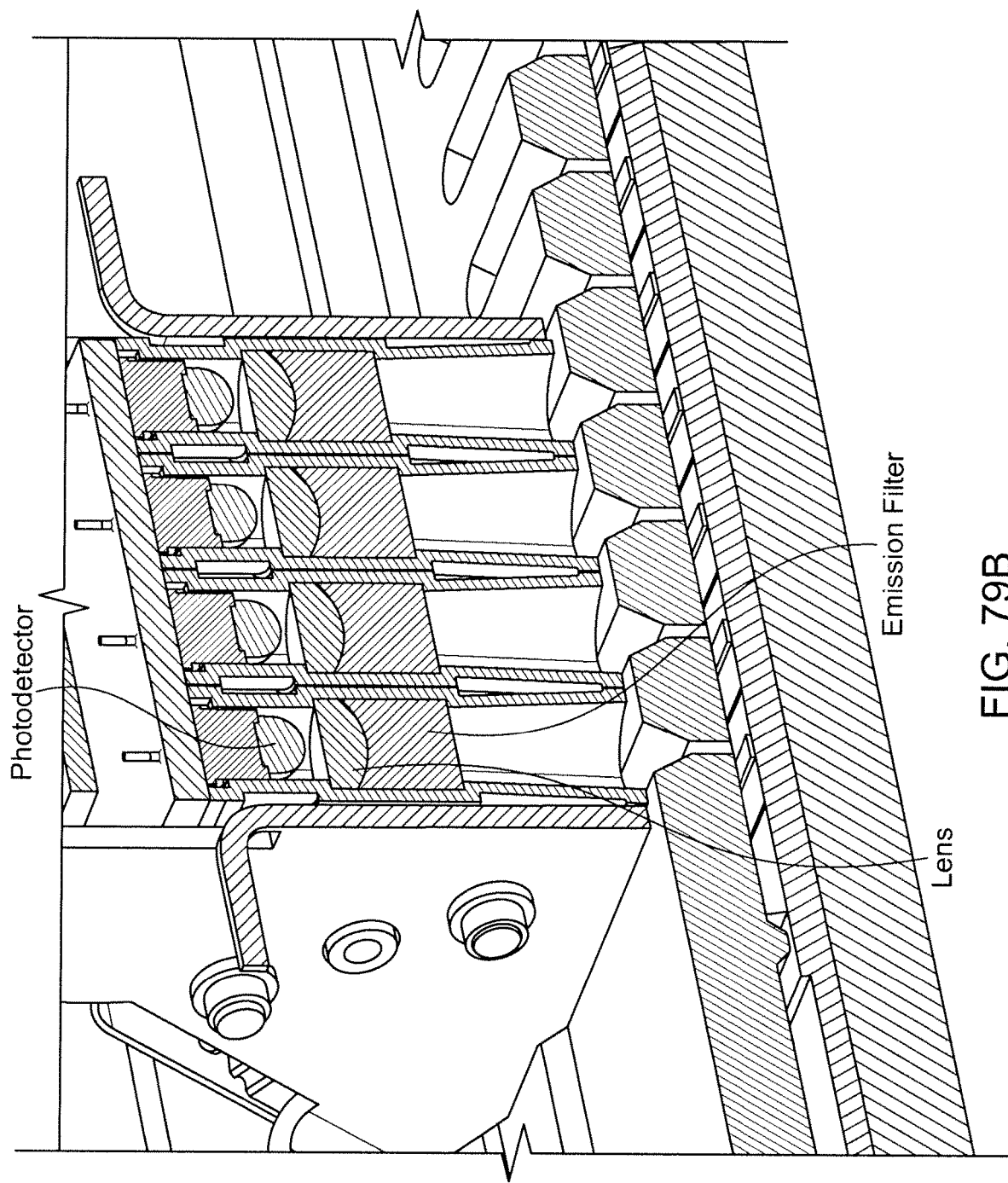
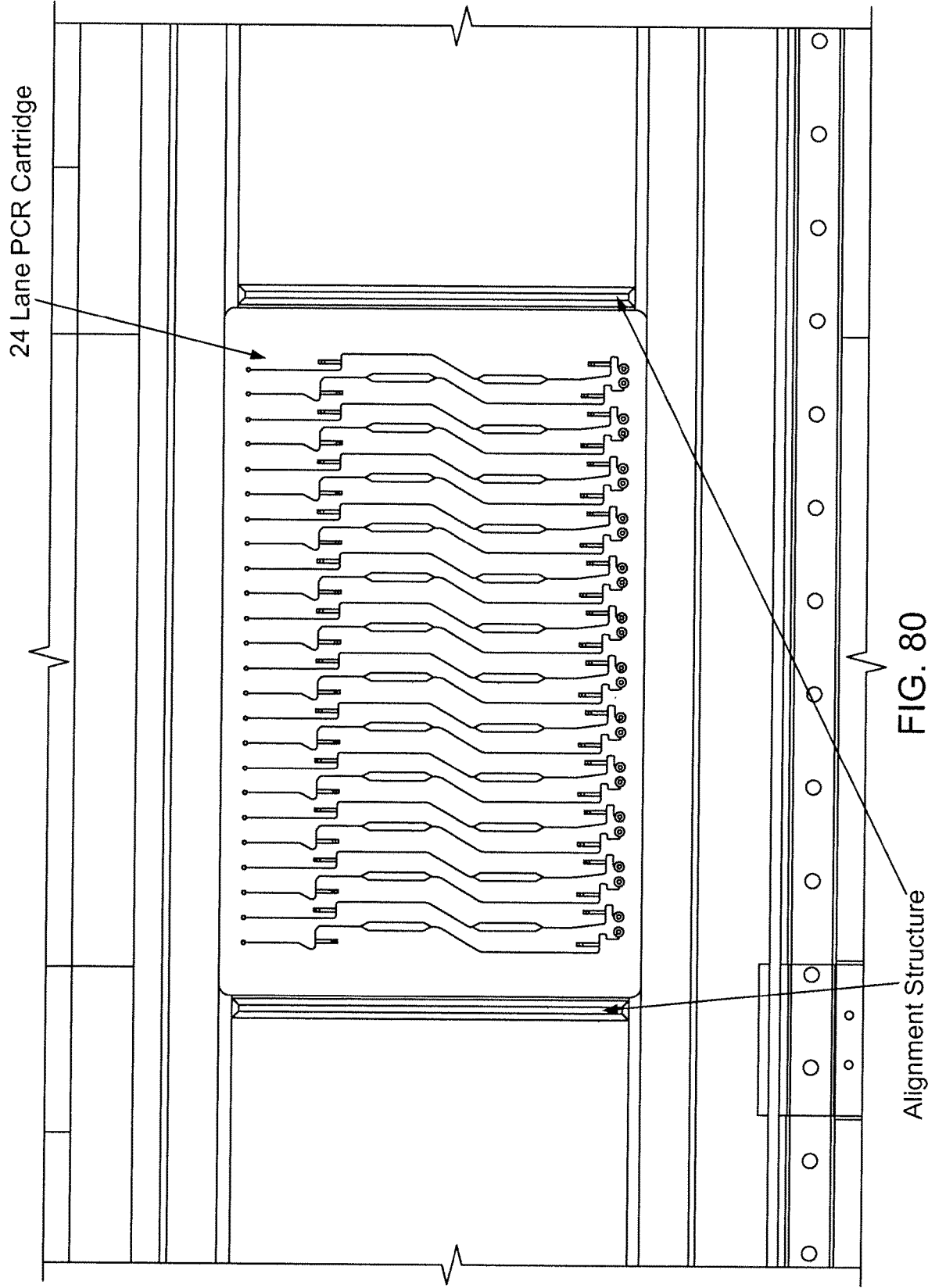


FIG. 79A





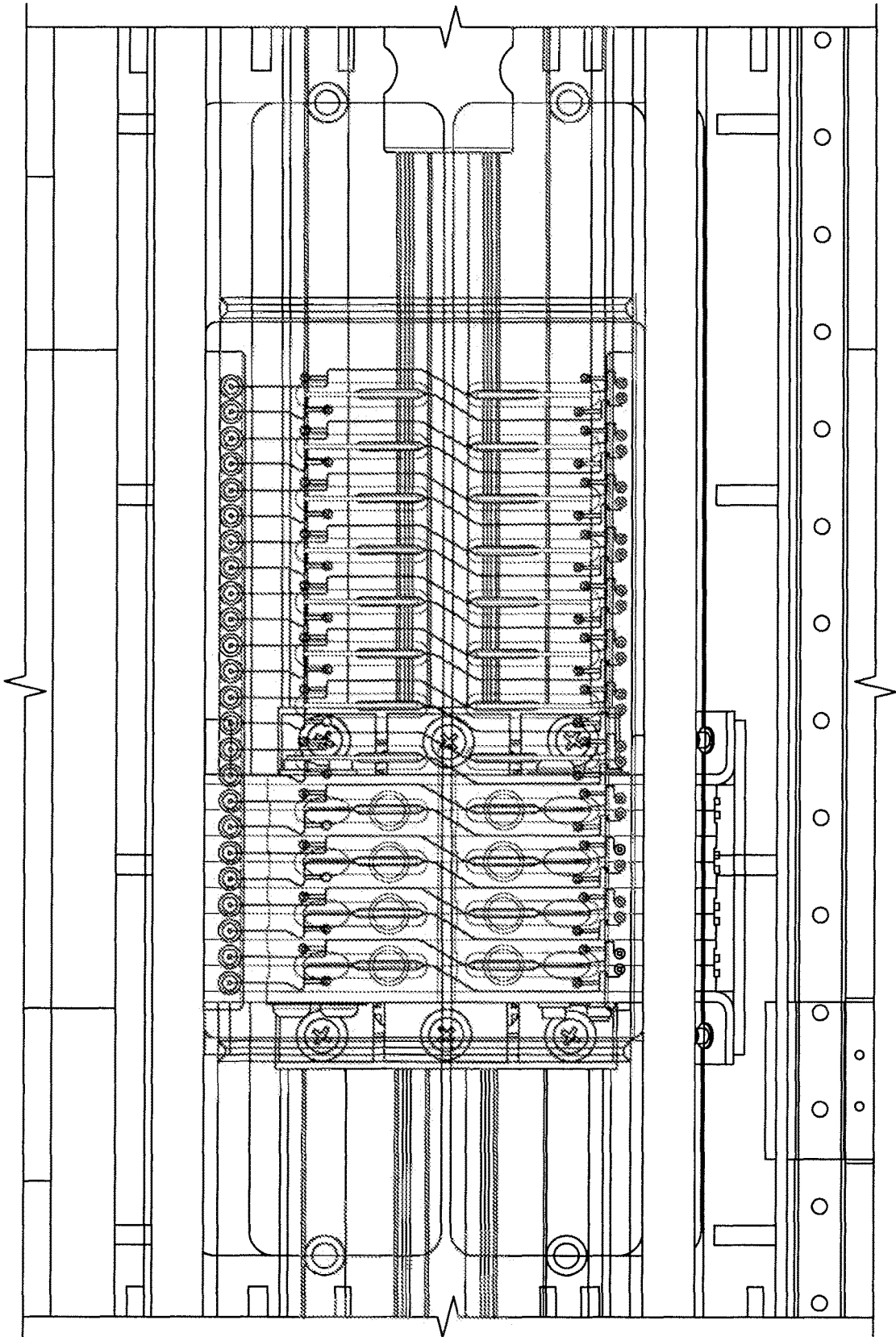


FIG. 81

U.S. Patent

Apr. 28, 2020

Sheet 111 of 121

US 10,632,466 B1

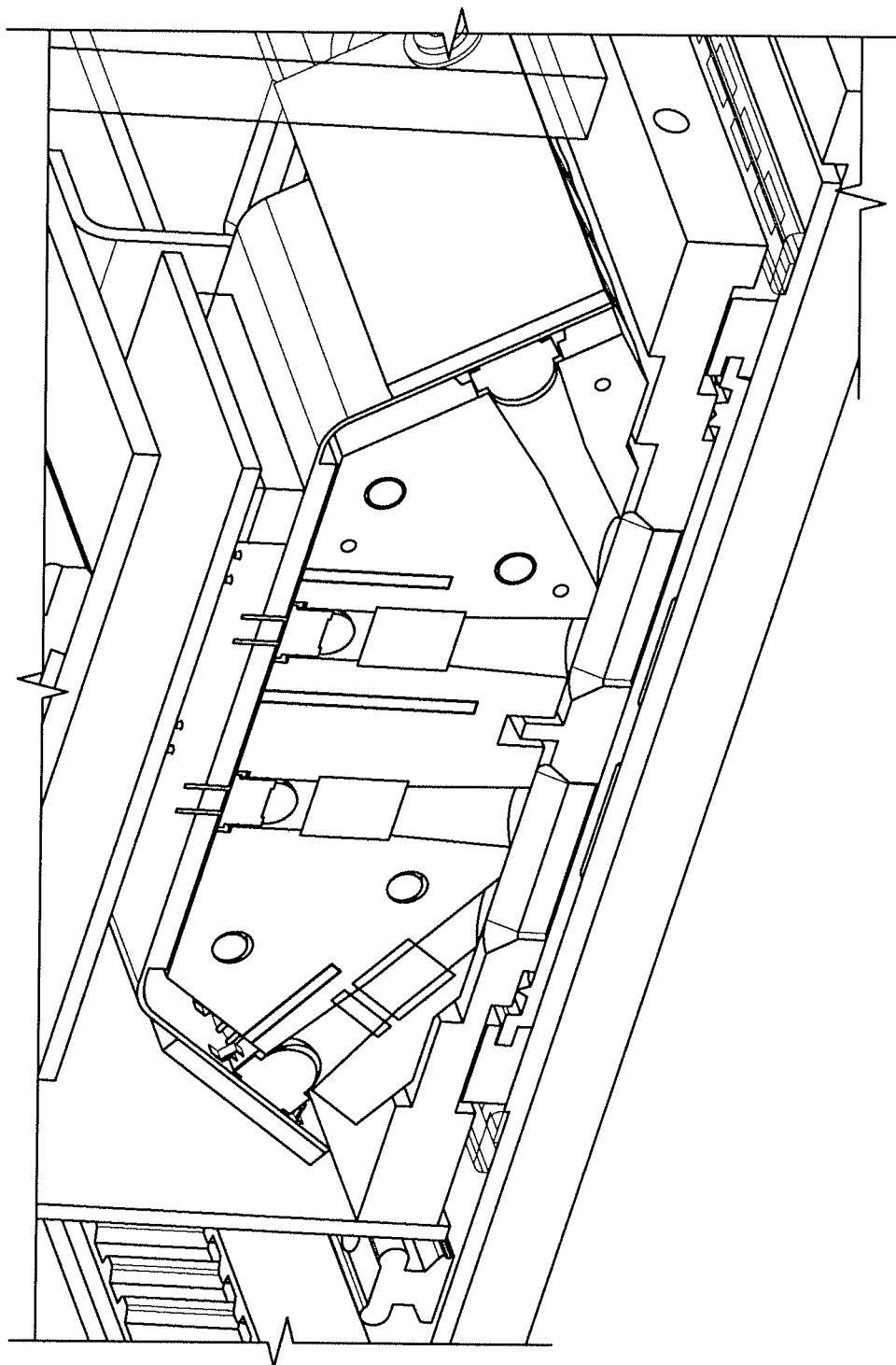


FIG. 82

U.S. Patent

Apr. 28, 2020

Sheet 112 of 121

US 10,632,466 B1

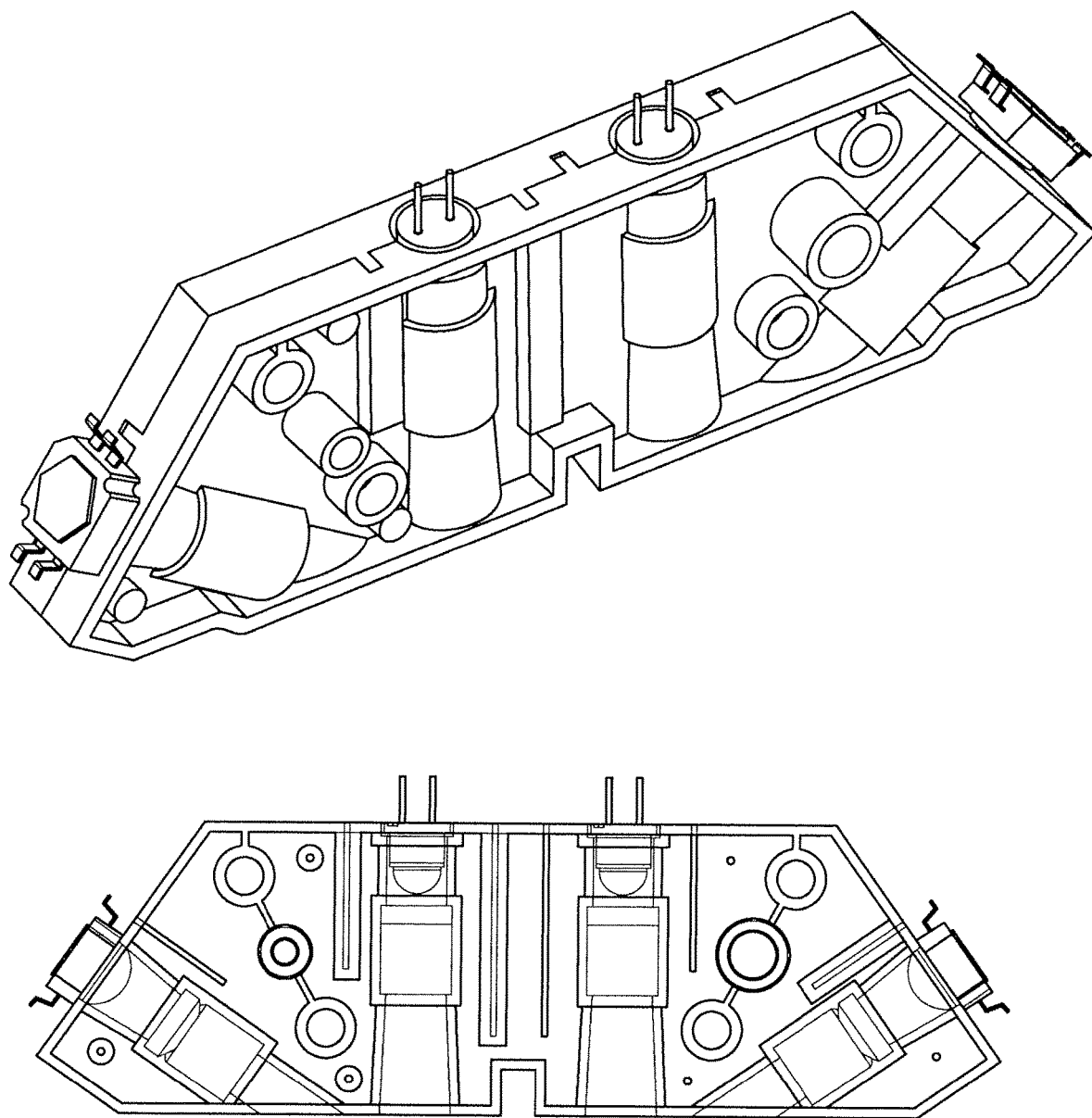


FIG. 83

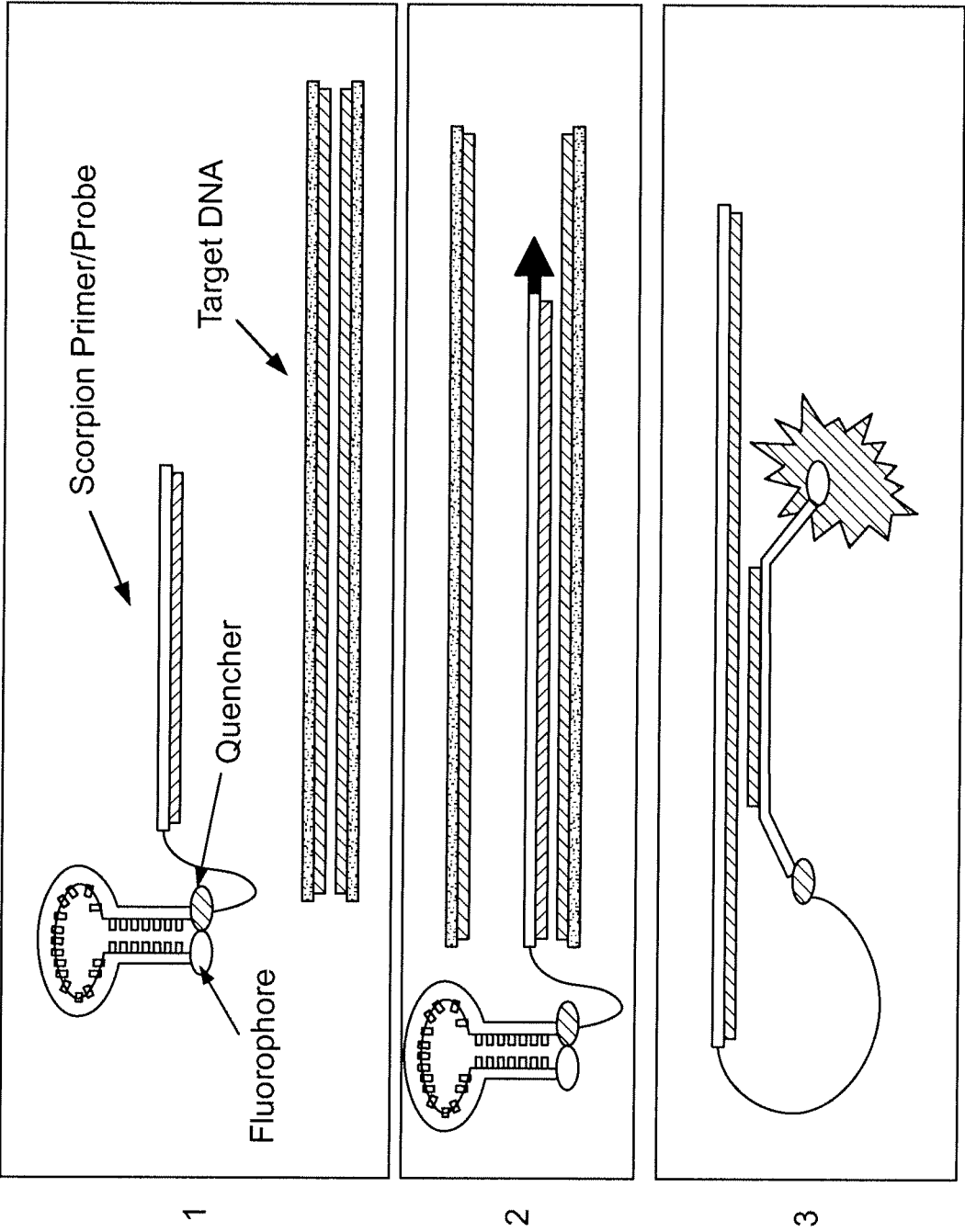


FIG. 84

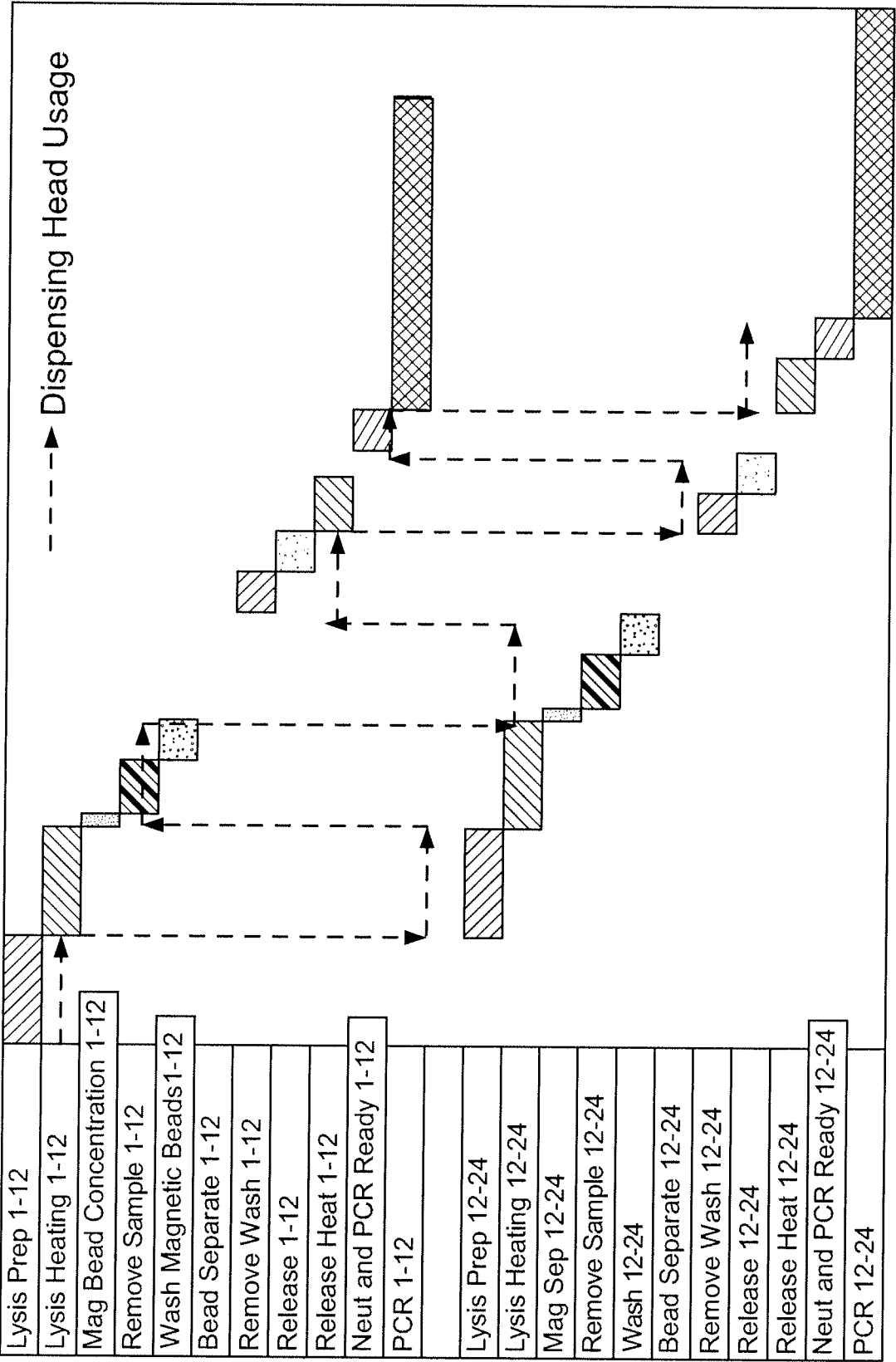


FIG. 85A

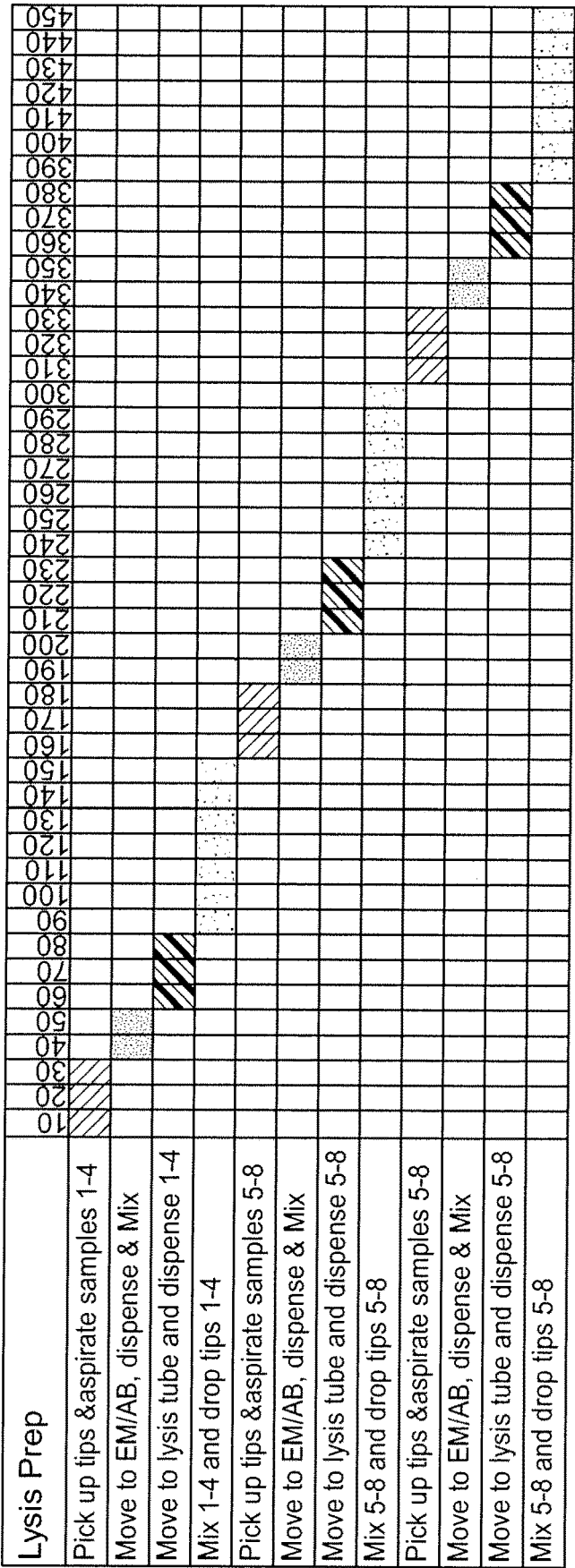


FIG. 85B

[illegible]

FIG. 85C

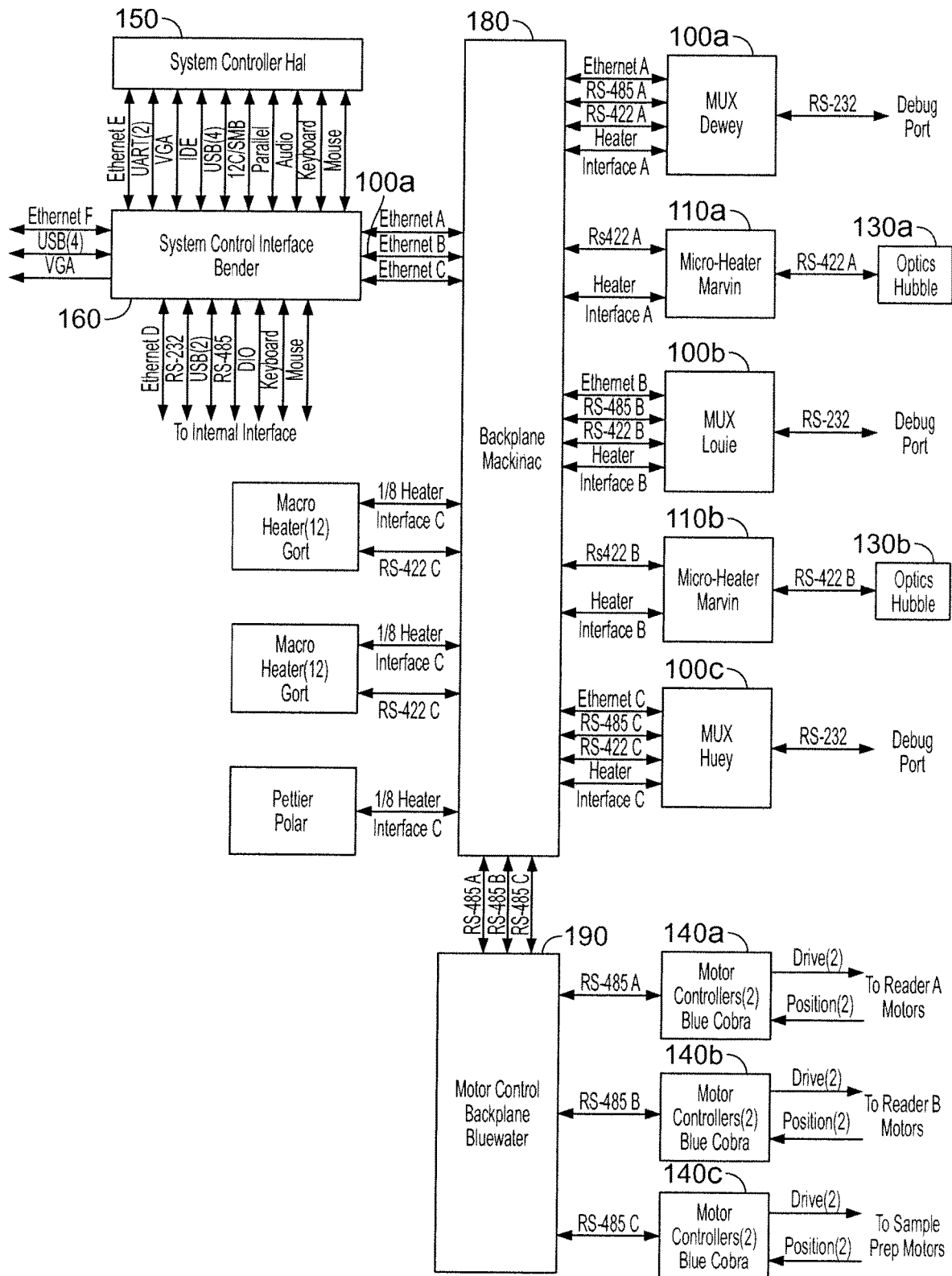


FIG. 86 Electronics Block Diagram

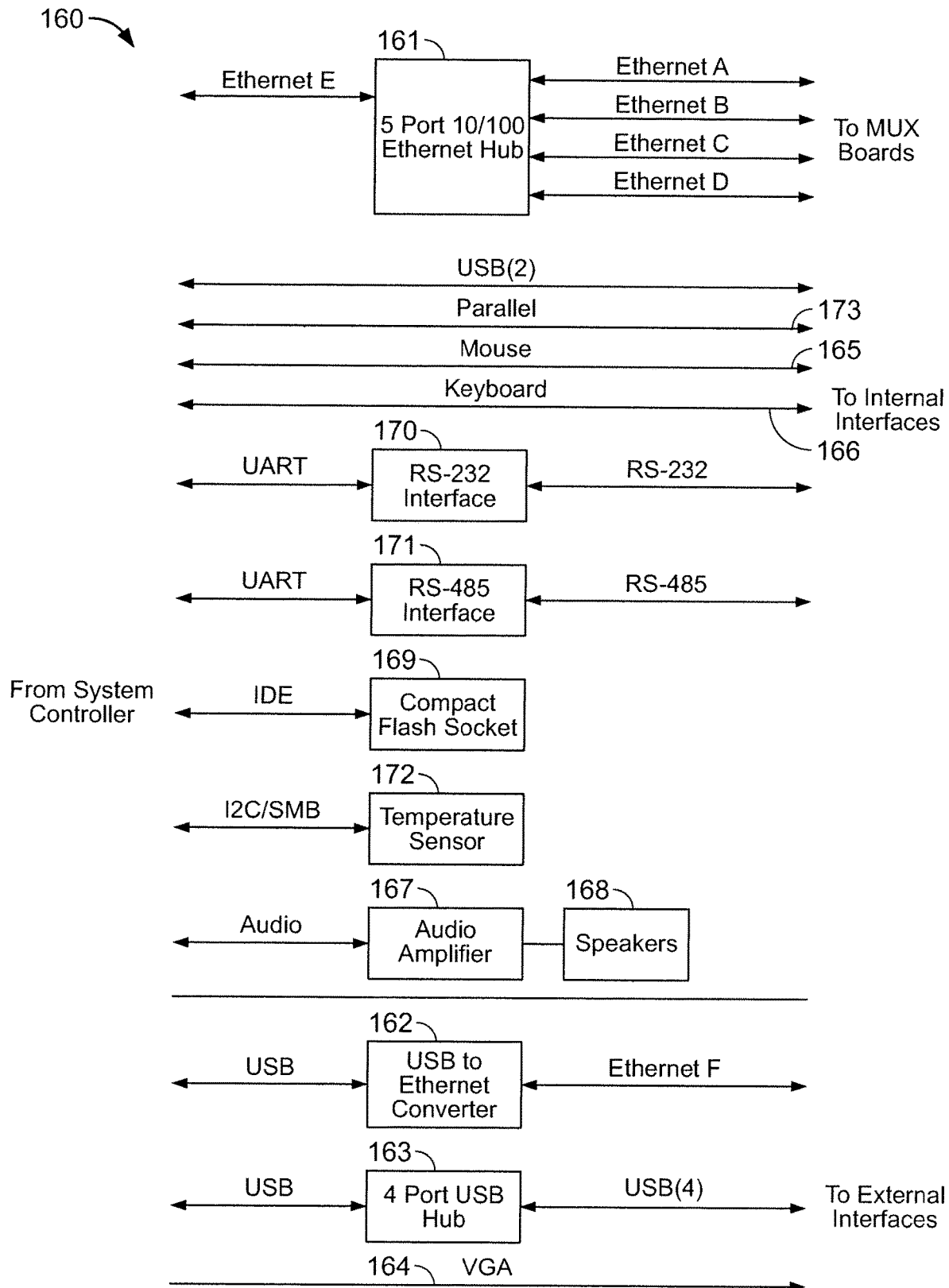


FIG. 87 Processor Base Board Block Diagram

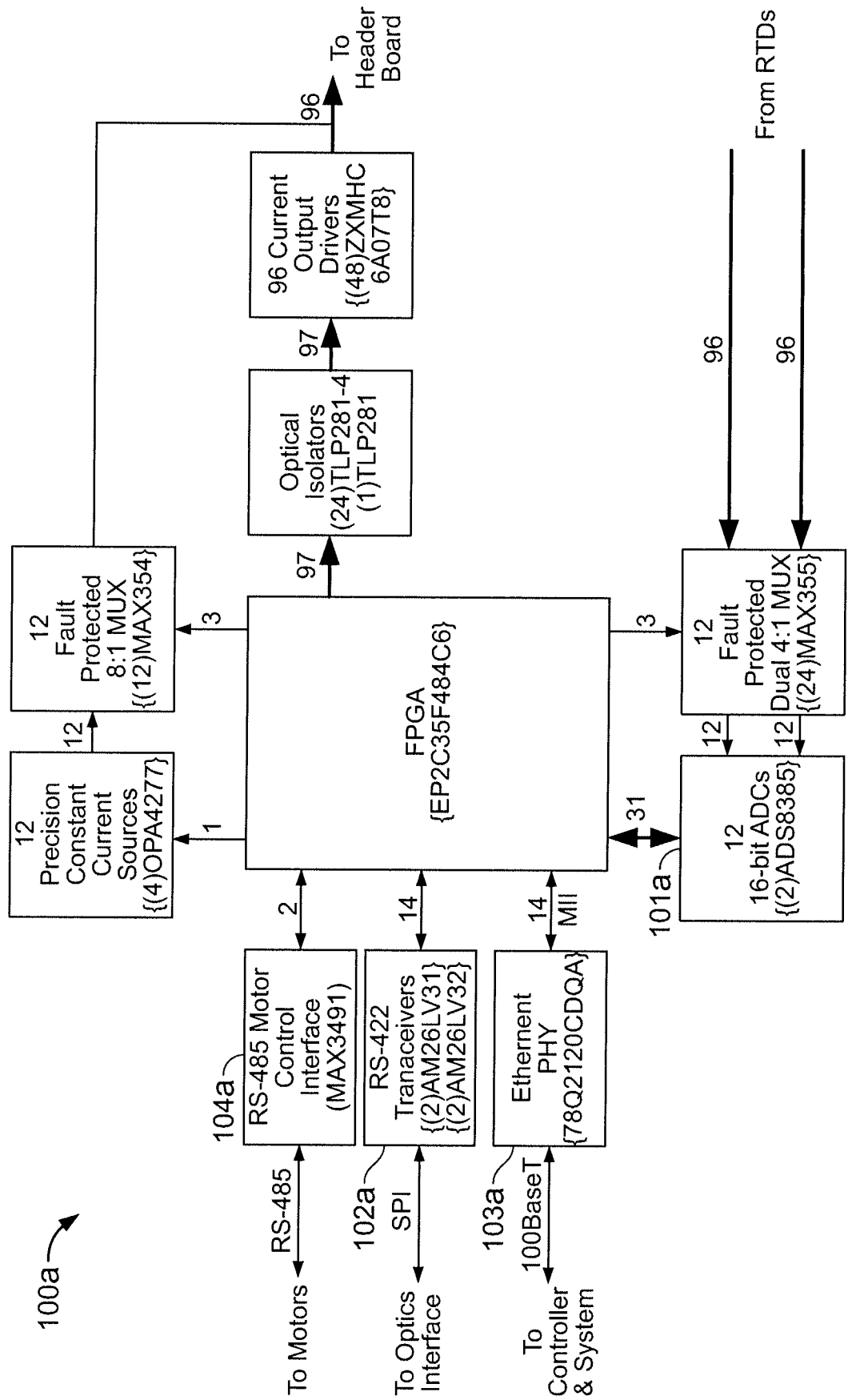


FIG. 88 MUX Board Block Diagram

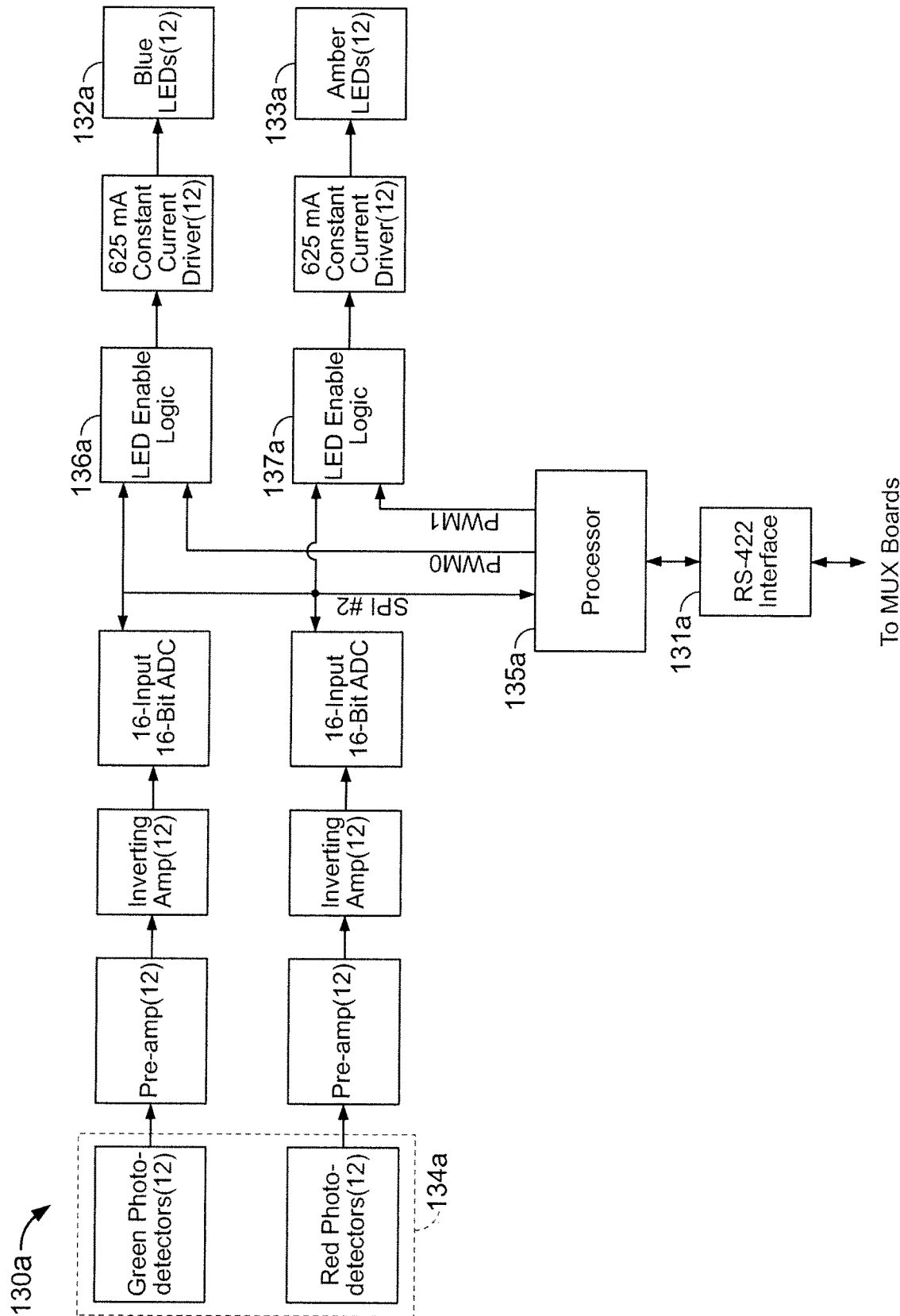


FIG. 89 MUX Board Block Diagram

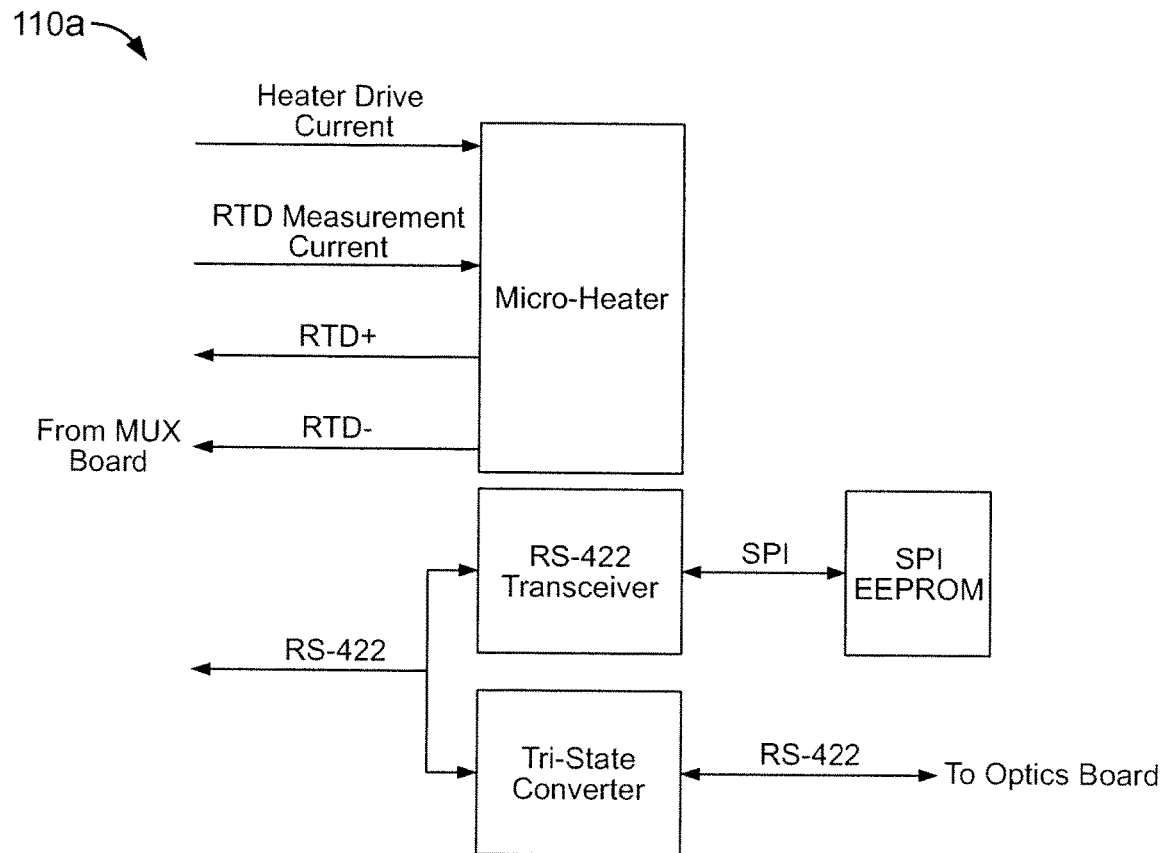


FIG. 90 Micro-Heater Board Block Diagram

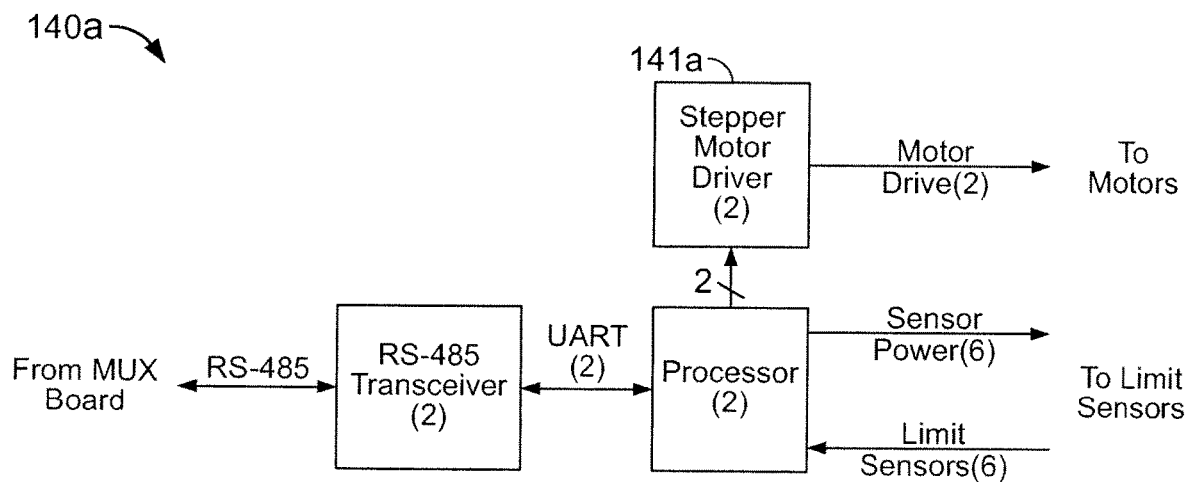


FIG. 91 Motor Control Board Block Diagram

US 10,632,466 B1

1

INTEGRATED APPARATUS FOR PERFORMING NUCLEIC ACID EXTRACTION AND DIAGNOSTIC TESTING ON MULTIPLE BIOLOGICAL SAMPLES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 16/124672, filed Sep. 7, 2018, which is a continuation of U.S. patent application Ser. No. 14/941,087, filed Nov. 13, 2015 and issued as U.S. Pat. No. 10,071,376 on Sep. 11, 2018, which is a continuation of U.S. patent application Ser. No. 12/218,498, filed Jul. 14, 2008 and issued as U.S. Pat. No. 9,186,677 on Nov. 17, 2015, which claims the benefit of priority under 35 U.S.C. §119(e) to U.S. Provisional Application No. 60/959,437, filed Jul. 13, 2007, and is a continuation-in-part of U.S. patent application Ser. No. 11/985,577, filed Nov. 14, 2007 and issued on Aug. 16, 2011 as U.S. Pat. No. 7,998,708. The disclosures of all of the above-referenced prior applications, publications, and patents are considered part of the disclosure of this application, and are incorporated by reference herein in their entirety.

TECHNICAL FIELD

The technology described herein generally relates to systems for extracting polynucleotides from multiple samples, particularly from biological samples, and additionally to systems that subsequently amplify and detect the extracted polynucleotides. The technology more particularly relates to microfluidic systems that carry out PCR on multiple samples of nucleotides of interest within microfluidic channels, and detect those nucleotides.

BACKGROUND

The medical diagnostics industry is a critical element of today's healthcare infrastructure. At present, however, in vitro diagnostic analyses no matter how routine have become a bottleneck in patient care. There are several reasons for this. First, many diagnostic analyses can only be done with highly specialist equipment that is both expensive and only operable by trained clinicians. Such equipment is found in only a few locations often just one in any given urban area. This means that most hospitals are required to send out samples for analyses to these locations, thereby incurring shipping costs and transportation delays, and possibly even sample loss or mishandling. Second, the equipment in question is typically not available 'on-demand' but instead runs in batches, thereby delaying the processing time for many samples because they must wait for a machine to fill up before they can be run.

Understanding that sample flow breaks down into several key steps, it would be desirable to consider ways to automate as many of these as possible. For example, a biological sample, once extracted from a patient, must be put in a form suitable for a processing regime that typically involves using PCR to amplify a vector (such as a nucleotide) of interest. Once amplified, the presence of a nucleotide of interest from the sample needs to be determined unambiguously. Preparing samples for PCR is currently a time-consuming and labor intensive step, though not one requiring specialist skills, and could usefully be automated. By contrast, steps such as PCR and nucleotide detection (or 'nucleic acid

2

testing') have customarily only been within the compass of specially trained individuals having access to specialist equipment.

There is a need for a method and apparatus of carrying out sample preparation on samples in parallel, with or without PCR and detection on the prepared biological samples, and preferably with high throughput, but in a manner that can be done routinely at the point of care, or without needing the sample to be sent out to a specialized facility.

The discussion of the background herein is included to explain the context of the inventions described herein. This is not to be taken as an admission that any of the material referred to was published, known, or part of the common general knowledge as at the priority date of any of the claims.

Throughout the description and claims of the specification the word "comprise" and variations thereof, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

SUMMARY

A diagnostic apparatus, comprising: a first module configured to extract nucleic acid simultaneously from a plurality of nucleic-acid containing samples, wherein the first module comprises: one or more racks, each configured to accept a number of samples and a corresponding number of holders, wherein each holder comprises a process chamber, a waste chamber, one or more pipette tips, and one or more receptacles, wherein the one or more receptacles contain respectively sufficient quantities of one or more reagents for carrying out extraction of nucleic acid from a sample; a magnetic separator configured to move relative to the process chambers of each holder; a heater assembly configured to independently heat each of the process chambers; and a liquid dispenser configured to carry out fluid transfer operations on two or more holders simultaneously; and a second module configured to simultaneously amplify the nucleic acid each configured to receive a microfluidic cartridge, wherein the cartridge is configured to separately accept and to separately amplify the nucleic acid extracted from multiple samples; and one or more detection systems.

A diagnostic apparatus comprising one or more racks, on each of which is mounted a number of nucleic acid containing samples and a corresponding number of holders, wherein each holder comprises a process chamber, a waste chamber, one or more pipette tips, and one or more receptacles, wherein the one or more receptacles contain, respectively, sufficient quantities of one or more reagents for carrying out extraction of nucleic acid from a sample; a magnetic separator movable from a first position to a second position adjacent to the process chamber of each of the one or more holders; a heater assembly comprising a number of heater units, each of which is in thermal contact with one of the process chambers; one or more bays, each bay having a shape of inlets each of which is in fluid communication with one of a number of channels in which nucleic acid extracted from one of the number of samples is amplified, and wherein the cartridge further comprises one or more windows that permit detection of amplified nucleic acid; a liquid dispenser having one or more dispensing heads, wherein the liquid dispenser is movable from a first position above a first holder to a second position above a second holder, and is movable from the first position above the first holder to a different position above the first holder, and is further movable from a position above one of the holders to a position above one

US 10,632,466 B1

3

of the number of inlets; and one or more detection systems positioned in proximity to the one or more windows.

A diagnostic instrument comprising: a liquid handling unit that extracts nucleic acid from a sample in a unitized reagent strip; a microfluidic cartridge that, in conjunction with a heater element, carries out real-time PCR on nucleic acid extracted from the sample, and a detector that provides a user with a diagnosis of whether the sample contains a nucleotide of interest.

Also described herein are methods of using the diagnostic apparatus, including a method of diagnosing a number of samples in parallel, using the apparatus.

A unitized reagent holder, comprising: a strip, to which is attached: a single process tube; one or more receptacles, each of which holding a reagent selected from the group consisting of: a sample preparation reagent, PCR reagents for a first analyte, and one or more liquid reagents; a waste tube; one or more sockets configured to hold one or more pipette tips; and a pipette tip sheath configured to surround the one or more pipette tips.

A liquid dispenser, comprising: one or more sensors; a manifold; one or more pumps in fluid communications with the manifold; one or more dispense heads in fluid communication with the manifold; a gantry that provides freedom of translational motion in three dimensions; and electrical connections that accept electrical signals from an external controller, wherein the liquid dispenser has no inlet or outlet for fluids; other than through the one or more pumps.

A separator for magnetic particles, comprising: one of more magnets aligned linearly; a motorized shaft upon which the one or more magnets can rise or fall in such a manner that the one or more magnets attains close proximity to one or more receptacles containing magnetic particles; and control circuitry to control motion of the motorized shaft.

An integrated separator and heater, comprising: a heater assembly, wherein the heater assembly comprises a plurality of independently controllable heater units, each of which is configured to accept and to heat a process chamber, one or more magnets aligned linearly; a motorized shaft upon which the one or more magnets can rise or fall in such a manner that the one or more magnets attains close proximity to one or more of the process chambers; and control circuitry to control motion of the motorized shaft and heating of the heater units.

A preparatory apparatus comprising: a first module configured to extract nucleic acid simultaneously from a number of nucleic-acid containing samples, wherein the first module comprises: one or more racks, each configured to accept the number of samples and a corresponding number of holders, wherein each holder comprises a process chamber, a waste chamber, one or more pipette tips, and one or more receptacles, wherein the one or more receptacles contain, respectively, sufficient quantities of one or more reagents for carrying out extraction of nucleic acid from a sample; a magnetic separator configured to move relative to the process chambers of each holder, a heater assembly configured to independently heat each of the process chambers; and a liquid dispenser configured to carry out fluid transfer operations on two or more holders simultaneously; and a second module configured to receive and to store the nucleic acid extracted from the number of samples.

A preparatory apparatus comprising: one or more racks, on each of which is mounted a number of nucleic acid containing samples and a corresponding number of holders, wherein each holder comprises a process chamber, a waste chamber, one or more pipette tips, and one or more recep-

4

tacles, wherein the one or more receptacles contain, respectively, sufficient quantities of one or more reagents for carrying out extraction of nucleic acid from a sample; a magnetic separator moveable from a first position to a second position adjacent to the process chambers of each holder; a heater assembly comprising a number of heater units, each of which is in contact with a process chamber; a liquid dispenser movable from a first position above a first holder to a second position above a second holder; and a storage compartment having a number of compartments, wherein each compartment stores the nucleic acid extracted from one of the number of samples.

A unitized reagent holder, comprising: a strip, to which is attached: a single process tube; one or more receptacles, each of which holding a reagent selected from the group consisting of: a sample preparation reagent, and one or more liquid reagents; a waste tube; one or more sockets configured to hold one or more pipette tips; and a pipette tip sheath configured to surround the one or more pipette tips.

The present technology additionally includes a process for extracting nucleic acid from multiple samples in parallel, using the apparatus as described herein.

BRIEF DESCRIPTION OF SELECTED DRAWINGS

FIG. 1A shows a schematic of a preparatory apparatus; FIG. 1B shows a schematic of a diagnostic apparatus;

FIG. 2 shows a schematic of control circuitry.

FIGS. 3A and 3B show exterior views of an exemplary apparatus.

FIG. 4 shows an exemplary interior view of an apparatus.

FIG. 5 shows perspective views of an exemplary rack for sample holders.

FIG. 6 shows perspective views of the rack of FIG. 5 in conjunction with a heater unit.

FIG. 7 shows a perspective view of an exemplary rack for sample holders.

FIG. 8A-8K show various views of the rack of FIG. 7.

FIG. 9 shows an area of an apparatus configured to accept a rack of FIG. 7.

FIGS. 10A and 10B show an first exemplary embodiment of a reagent holder having a pipette sheath, in perspective view (FIG. 10A) and underside view (FIG. 10B).

FIG. 11 shows an exemplary embodiment of a reagent holder not having a pipette sheath, in perspective view.

FIGS. 12A-12C show a second exemplary embodiment of a reagent holder having a pipette sheath, in perspective view (FIG. 12A) and cross-sectional view (FIG. 12B), and exploded view (FIG. 12C).

FIGS. 13A and 13B show a stellated feature on the interior of a reagent tube, in cross-sectional (FIG. 13A) and plan (FIG. 13B) view.

FIG. 14 shows a sequence of pipetting operations in conjunction with a reagent tube having a stellated feature.

FIG. 15 shows embodiments of a laminated layer.

FIG. 16 shows a sequence of pipetting operations in conjunction with a laminated layer.

FIGS. 17A-17D show an exemplary kit containing holders and reagents.

FIG. 18 shows a liquid dispense head.

FIGS. 19A-19C show a liquid dispense head.

FIG. 20 shows an exemplary distribution manifold.

FIG. 21 shows a scanning read-head attached to a liquid dispense head.

FIG. 22 shows a barcode scanner in cross-sectional view.

US 10,632,466 B1

5

FIG. 23 shows a barcode reader positioned above a microfluidic cartridge.

FIG. 24 shows pipette tip sensors.

FIGS. 25A-25B show an exemplary device for stripping pipette tip.

FIG. 26 shows a heater unit in perspective and cross-sectional view.

FIG. 27 shows an integrated heater and separator unit in cross-sectional view.

FIG. 28 shows a cartridge auto-loader.

FIG. 29 shows a cartridge stacker.

FIG. 30 shows a cartridge stacker in position to deliver a cartridge to an auto-loader.

FIG. 31 shows a cartridge loading system.

FIG. 32 shows a disposal unit for used cartridges.

FIG. 33 shows a cartridge stacker in full and empty configurations.

FIG. 34 shows a microfluidic cartridge, a read-head, and a cartridge tray.

FIG. 35 shows a cross-section of a pipetting head and a cartridge in position in a microfluidic apparatus.

FIG. 36 shows an exemplary microfluidic cartridge having a 3-layer construction.

FIG. 37 shows a plan of microfluidic circuitry and inlets in an exemplary multi-lane cartridge;

FIG. 38A shows an exemplary multi-lane cartridge.

FIG. 38B shows a portion of an exemplary multi-lane cartridge.

FIGS. 39A-39C show diagrams of exemplary microfluidic valves. FIG. 40A additionally shows the valve in an open state, and the valve in a closed state.

FIG. 41 shows a vent.

FIG. 42 shows an exemplary highly-multiplexed microfluidic cartridge;

FIGS. 43-46 show various aspects of exemplary highly multiplexed microfluidic cartridge; and

FIGS. 47A-C show various aspects of a radially configured highly multiplexed microfluidic cartridge.

FIG. 48 shows a view in cross-section of a microfluidic cartridge.

FIGS. 49A, 49B show a PCR reaction chamber and associated heaters.

FIGS. 51A-52C shows various cut-away sections that can be used to improve cooling rates during PCR thermal cycling.

FIG. 52 shows a plot of temperature against time during a PCR process, as performed on a microfluidic cartridge as described herein.

FIG. 53 shows an assembly process for a cartridge as further described herein.

FIGS. 54A and 54B show exemplary apparatus for carrying out wax deposition.

FIGS. 55A and 55B show exemplary deposition of wax droplets into microfluidic valves.

FIG. 56 shows an overlay of an array of heater elements on an exemplary multi-lane microfluidic cartridge, wherein various microfluidic networks are visible.

FIG. 57 shows a cross-sectional view of an exemplary detector.

FIG. 58 shows a perspective view of a detector in a read-head.

FIG. 59 shows a cutaway view of an exemplary detector in a read-head.

FIG. 60 shows an exterior view of an exemplary multiplexed read-head with an array of detectors therein.

FIG. 61 shows an cutaway view of an exemplary multiplexed read-head with an array of detectors therein.

6

FIG. 62 shows a block diagram of exemplary electronic circuitry in conjunction with a detector as described herein.

FIG. 63 shows an exemplary liquid dispensing system.

FIG. 64 shows an exemplary heater/separator.

FIGS. 65A and 65B show exemplary aspects of a computer-based user interface.

FIG. 66 shows schematically layout of components of a preparatory apparatus.

FIG. 67 shows layout of components of an exemplary preparatory apparatus.

FIG. 68 shows schematically layout of components of a diagnostic apparatus.

FIG. 69 shows layout of components of an exemplary diagnostic apparatus.

FIGS. 70 and 71 show exterior and interior of an exemplary diagnostic apparatus.

FIGS. 72A and 72B show a thermocycling unit configured to accept a microfluidic cartridge.

FIG. 73 shows schematically a layout of components of a high-efficiency diagnostic apparatus.

FIG. 74 shows layout of components of an exemplary high-efficiency diagnostic apparatus.

FIG. 75 shows a plan view of a 24-lane microfluidic cartridge.

FIG. 76 shows a perspective view of the cartridge of FIG. 73.

FIG. 77 shows an exploded view of the cartridge of FIG. 75.

FIG. 78 shows an exemplary detection unit.

FIGS. 79A, 79B show cutaway portions of the detection unit of FIG. 78

FIGS. 80 and 81 show alignment of the detection unit with a microfluidic cartridge.

FIGS. 82 and 83 show exterior and cutaways, respectively, of an optics block.

FIG. 84 shows a Scorpion reaction, schematically.

FIGS. 85A-85C show, schematically, pipette head usage during various preparatory processes.

FIGS. 86-91 show exemplary layouts of electronics control circuitry.

DETAILED DESCRIPTION

Nucleic acid testing (NAT) as used herein is a general term that encompasses both DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) testing. Exemplary protocols that are specific to RNA and to DNA are described herein. It is to be understood that generalized descriptions where not specific to RNA or to DNA either apply to each equally or can be readily adapted to either with minor variations of the description herein as amenable to on of ordinary skill in the art. It is also to be understood that the terms nucleic acid and polynucleotide may be used interchangeably herein.

The apparatuses as described herein therefore find application to analyzing any nucleic acid containing sample for any purpose, including but not limited to genetic testing, and clinical testing for various infectious diseases in humans. Targets for which clinical assays currently exist, and that may be tested for using the apparatus and methods herein may be bacterial or viral, and include, but are not limited to: Chlamydia Trachomatis (CT); Neisseria Gonorrhea (GC); Group B Streptococcus; HSV; HSV Typing; CMV; Influenza A & B; MRSA; RSV; TB; Trichomonas; Adenovirus; Bordetella; BK; JC; HHV6; EBV; Enterovirus; and *M. pneumoniae*.

The apparatus herein can be configured to run on a laboratory benchtop, or similar environment, and can text

US 10,632,466 B1

7

approximately 45 samples per hour when run continuously throughout a normal working day. This number can be increased, according to the number of tests that can be accommodated in a single batch, as will become clear from the description herein. Results from individual raw samples are typically available in less than 1 hour.

Where used herein, the term “module” should be taken to mean an assembly of components, each of which may have separate, distinct and/or independent functions, but which are configured to operate together to produce a desired result or results. It is not required that every component within a module be directly connected or in direct communication with every other. Furthermore, connectivity amongst the various components may be achieved with the aid of a component, such as a processor, that is external to the module.

Apparatus Overview

An apparatus having various components as further described herein can be configured into at least two formats, preparatory and diagnostic, as shown respectively in FIGS. 1A and 1B. A schematic overview of a preparatory apparatus **981** for carrying out sample preparation as further described herein is shown in FIG. 1A. An overview of a diagnostic apparatus **971** is shown in FIG. 1B. The geometric arrangement of the components of systems **971**, **981** shown in FIGS. 1A and 1B is exemplary and not intended to be limiting.

A processor **980**, such as a microprocessor, is configured to control functions of various components of the system as shown, and is thereby in communication with each such component requiring control. It is to be understood that many such control functions can optionally be carried out manually, and not under control of the processor. Furthermore, the order in which the various functions are described, in the following, is not limiting upon the order in which the processor executes instructions when the apparatus is operating. Thus, processor **980** can be configured to receive data about a sample to be analyzed, e.g., from a sample reader **990**, which may be a barcode reader, an optical character reader, or an RFID scanner (radio frequency tag reader). It is also to be understood that, although a single processor **980** is shown as controlling all operations of apparatus **971** and **981**, such operations may be distributed, as convenient, over more than one processor.

Processor **980** can be configured to accept user instructions from an input **984**, where such instructions may include instructions to start analyzing the sample, and choices of operating conditions. Although not shown in FIGS. 1A and 1B, in various embodiments, input **984**, can include one or more input devices selected from the group consisting of: a keyboard, a touch-sensitive surface, a microphone, a track-pad, a retinal scanner, a holographic projection of an input device, and a mouse. A suitable input device may further comprise a reader of formatted electronic media, such as, but not limited to, a flash memory card, memory stick, USB-stick, CD, or floppy diskette. An input device may further comprise a security feature such as a fingerprint reader, retinal scanner, magnetic strip reader, or bar-code reader, for ensuring that a user of the system is in fact authorized to do so, according to pre-loaded identifying characteristics of authorized users. An input device may additionally—and simultaneously—function as an output device for writing data in connection with sample analysis. For example, if an input device is a reader of formatted electronic media, it may also be a writer of such media. Data that may be written to such media by such a device includes, but is not limited to, environmental information, such as

8

temperature or humidity, pertaining to an analysis, as well as a diagnostic result, and identifying data for the sample in question.

Processor **980** can be also configured to communicate with a display **982**, so that, for example, information about an analysis is transmitted to the display and thereby communicated to a user of the system. Such information includes but is not limited to: the current status of the apparatus; progress of PCR thermocycling; and a warning message in case of malfunction of either system or cartridge. Additionally, processor **980** may transmit one or more questions to be displayed on display **982** that prompt a user to provide input in response thereto. Thus, in certain embodiments, input **984** and display **982** are integrated with one another.

Processor **980** can be optionally further configured to transmit results of an analysis to an output device such as a printer, a visual display, a display that utilizes a holographic projection, or a speaker, or a combination thereof.

Processor **980** can be still further optionally connected via a communication interface such as a network interface to a computer network **988**. The communication interface can be one or more interfaces selected from the group consisting of: a serial connection, a parallel connection, a wireless network connection, a USB connection, and a wired network connection. Thereby, when the system is suitably addressed on the network, a remote user may access the processor and transmit instructions, input data, or retrieve data, such as may be stored in a memory (not shown) associated with the processor, or on some other computer-readable medium that is in communication with the processor. The interface may also thereby permit extraction of data to a remote location, such as a personal computer, personal digital assistant, or network storage device such as computer server or disk farm. The apparatus may further be configured to permit a user to e-mail results of an analysis directly to some other party, such as a healthcare provider, or a diagnostic facility, or a patient.

Additionally, in various embodiments, the apparatus can further comprise a data storage medium configured to receive data from one or more of the processor, and input device, and a communication interface, the data storage medium being one or more media selected from the group consisting of a hard disk drive, an optical disk drive, a flash card, and a CD-Rom.

Processor **980** can be further configured to control various aspects of sample preparation and diagnosis, as follows in overview, and as further described in detail herein. In FIGS. 1A and 1B, the apparatus **981** (or **971**) is configured to operate in conjunction with a complementary rack **970**. The rack is itself configured, as further described herein, to receive a number of biological samples **996** in a form suitable for work-up and diagnostic analysis, and a number of holders **972** that are equipped with various reagents, pipette tips and receptacles. The rack is configured so that, during sample work-up, samples are processed in the respective holders, the processing including being subjected, individually, to heating and cooling via heater assembly **977**. The heating functions of the heater assembly can be controlled by the processor **980**. Heater assembly **977** operates in conjunction with a separator **978**, such as a magnetic separator, that also can be controlled by processor **980** to move into and out of close proximity to one or more processing chambers associated with the holders **972**, wherein particles such as magnetic particles are present.

Liquid dispenser **976**, which similarly can be controlled by processor **980**, is configured to carry out various suck and dispense operations on respective sample, fluids and

US 10,632,466 B1

9

reagents in the holders **972**, to achieve extraction of nucleic acid from the samples. Liquid dispenser **976** can carry out such operations on multiple holders simultaneously. Sample reader **990** is configured to transmit identifying indicia about the sample, and in some instances the holder, to processor **980**. In some embodiments a sample reader is attached to the liquid dispenser and can thereby read indicia about a sample above which the liquid dispenser is situated. In other embodiments the sample reader is not attached to the liquid dispenser and is independently movable, under control of the processor. Liquid dispenser **976** is also configured to take aliquots of fluid containing nucleic acid extracted from one or more samples and direct them to storage area **974**, which may be a cooler. Area **974** contains, for example, a PCR tube corresponding to each sample. In other embodiments, there is not a separate Area **974**, but a cooler can be configured to cool the one or more holders **972** so that extracted nucleic acid is cooled and stored in situ rather than being transferred to a separate location.

FIG. 1B shows a schematic embodiment of a diagnostic apparatus **971**, having elements in common with apparatus **981** FIG. 1A but, in place of a storage area **974**, having a receiving bay **992** in which a cartridge **994** is received. The receiving bay is in communication with a heater **998** that itself can be controlled by processor **980** in such a way that specific regions of the cartridge are heated at specific times during analysis. Liquid dispenser **976** is thus configured to take aliquots of fluid containing nucleic acid extracted from one or more samples and direct them to respective inlets in cartridge **994**. Cartridge **994** is configured to amplify, such as by carrying out PCR, on the respective nucleic acids. The processor is also configured to control a detector **999** that receives an indication of a diagnosis from the cartridge **994**. The diagnosis can be transmitted to the output device **986** and/or the display **982**, as described hereinabove.

A suitable processor **980** can be designed and manufactured according to, respectively, design principles and semiconductor processing methods known in the art.

Embodiments of the apparatuses shown in outline in FIGS. 1A and 1B, its with other exemplary embodiments described herein, is advantageous because they do not require locations within the apparatus suitably configured for storage of reagents. Neither do embodiments of the system, or other exemplary embodiments herein, require inlet or outlet ports that are configured to receive reagents from, e.g., externally stored containers such as bottles, canisters, or reservoirs. Therefore, the apparatuses in FIGS. 1A and 1B are self-contained and operate in conjunction with holders **972**, wherein the holders are pre-packaged with reagents, such as in locations within it dedicated to reagent storage.

The apparatuses of FIGS. 1A and 1B may be configured to carry out operation in a single location, such as a laboratory setting, or may be portable so that they can accompany, e.g., physician, or other healthcare professional, who may visit patients at different locations. The apparatuses are typically provided with a power-cord so that they can accept AC power from a mains supply or generator. An optional transformer (not shown) built into each apparatus, or situated externally between a power socket and the system, transforms AC input power into a DC output for use by the apparatus. The apparatus may also be configured to operate by using one or more batteries and therefore is also typically equipped with a battery recharging system, and various warning devices that alert a user if battery power is becoming too low to reliably initiate or complete a diagnostic analysis.

10

The apparatuses of FIGS. 1A and 1B may further be configured, in other embodiment for multiplexed sample analysis and/or analysis of multiple batches of samples, where, e.g., a single rack holds a single batch of samples. In one such configuration, instances of a system, as outlined in FIG. 1B, accept and to process multiple microfluidic cartridges **994**. Each component shown in FIGS. 1A and 1B may therefore be present as many times as there are batches of samples, though the various components may be configured in a common housing.

In still another configuration, a system is configured to accept and to process multiple cartridges, but one or more components in FIGS. 1A and 1B is common to multiple cartridges. For example, a single apparatus may be configured with multiple cartridge receiving bays, but a common processor, detector, and user interface suitably configured to permit concurrent, consecutive, or simultaneous, control of the various cartridges. It is further possible that such an embodiment, also utilizes a single sample reader, and a single output device.

In still another configuration, a system as shown in FIG. 1B is configured to accept a single cartridge, wherein the single cartridge is configured to process more than 1. for example, 2, 3, 4, 5, or 6, samples in parallel, and independently of one another. Exemplary technology for creating cartridges that can handle multiple samples is described elsewhere, e.g., in U.S. application Ser. No. 60/859,284, incorporated herein by reference.

It is further consistent with the present technology that a cartridge can be tagged, e.g., with a molecular bar-code indicative of the sample, to facilitate sample tracking, and to minimize risk of sample mix-up. Methods for such tagging are described elsewhere, e.g., in U.S. patent application publication NO. 10/360,854, incorporated herein by reference.

Control electronics **840** implemented into apparatus **971** or **981**, shown schematically in the block diagram in FIG. 2, can include one or more functions in various embodiments, for example, for main control **900**, multiplexing **902**, display control **904**, detector control **906**, and the like. The main control function may serve as the hub of control electronics **840** in the apparatuses of FIGS. 1A and 1B, and can manage communication and control of the various electronic functions. The main control function can also support electrical and communications interface **908** with a user or an output device such as a printer **920**, as well as optional diagnostic and safety functions. In conjunction with main control function **900**, multiplexer function **902** can control sensor data **914** and output current **916** to help control heater assembly **977**. The display control function **904** can control output to and, if applicable, interpret input from touch screen LCD **846**, which can thereby provide a graphical interface to the user in certain embodiments. The detector function **906** can be implemented in control electronics **840** using typical control and processing circuitry to collect, digitize, filter, and/or transmit the data from a detector **999** such as one or more fluorescence detectors. Additional functions, not shown in FIG. 2, include but are not limited to control functions for controlling elements in FIGS. 1A and 1B such as a liquid dispense head, a separator, a cooler, and to accept data from a sample reader.

An exemplary apparatus, having functions according to FIGS. 1A or 1B, is shown in FIGS. 3A and 3B. The exemplary apparatus in FIGS. 3A and 3B has a housing **985**, and a cover **987**, shown in a closed position in FIG. 3A, and in an open position in FIG. 3B to reveal interior features **995**. Cover **987** optionally has a handle **989**, shown as oval and

US 10,632,466 B1

11

raised from the surface of the cover, but which may be other shapes such as square, rectangular, or circular, and which may be recessed in, or flush with, the surface of the cover. Cover **987** is shown as having a hinge, though other configurations such as a sliding cover are possible. Bumper **991** serves to prevent the cover from falling too far backwards and/or provides a point that holds cover **987** steady in an open position. Housing **985** is additionally shown as having one or more communications ports **983**, and one or more power ports **993**, which may be positioned elsewhere, such as on the rear of the instrument.

The apparatus of FIGS. **1A** and **1B** may optionally comprise one or more stabilizing feet that cause the body of the device to be elevated above a surface on which system **100** is disposed, thereby permitting ventilation underneath system **100**, and also providing a user with an improved ability to lift system **100**. There may be 2, 3, 4, 5, or 6, or more feet, depending upon the size of system **100**. Such feet are preferably made of rubber, or plastic, or metal, and in some embodiments may elevate the body of system **100** by from about 2 to about 10 mm above a surface on which it is situated.

FIG. **4** shows an exemplary configuration of a portion of an interior of an exemplary apparatus, such as that shown in FIGS. **3A** and **3B**. In FIG. **4** are shown a rack **970**, containing a number of reagent holders **972** and patient samples **996**, as well as, in close proximity thereto, a receiving bay **992** having a cartridge **994**, for performing PCR on polynucleotides extracted from the samples.

Rack

The apparatus further comprises one or more racks configured to be insertable into, and removable from, the apparatus, each of the racks being further configured to receive a plurality of reagent holders, and to receive a plurality of sample tubes, wherein the reagent holders are in one-to-one correspondence with the sample tubes, and wherein the reagent holders each contain sufficient reagents to extract polynucleotides from a sample and place the polynucleotides into a PCR-ready form. Exemplary reagent holders are further described elsewhere herein.

An apparatus may comprise 1, 2, 3, 4, or 6 racks, and each rack may accept 2, 4, 6, 8, 10, 12, 16, or 20 samples such as in sample tubes **802**, and a corresponding number of holders **804**, each at least having one or more pipette tips, and one or more containers for reagents.

A rack is typically configured to accept a number of reagent holders **804**, such as those further described herein, the rack being configured to hold one or more such holders, either permitting access on a laboratory benchtop to reagents stored in the holders, or situated in a dedicated region of the apparatus permitting the holders to be accessed by one or more other functions of the apparatus, such as automated pipetting, heating of the process tubes, and magnetic separating of affinity beads.

Two perspective views of an exemplary rack **800**, configured to accept 12 sample tubes and 12 corresponding reagent holders, in 12 lanes, are shown in FIG. **5**. A lane, as used herein in the context of a rack, is a dedicated region of the rack designed to receive a sample tube and corresponding reagent holder. Two perspective views of the same exemplary rack, in conjunction with a heater unit, are shown in FIG. **6**.

Various views of a second exemplary rack **800**, also configured to accept 12 sample tubes and 12 reagent holders, are shown in FIG. **7**, and FIGS. **8A-8K**. Thus, the following views are shown: side plan (FIG. **8A**); front plan, showing sample tubes (FIG. **8B**); rear plan, showing reagent holders

12

(FIG. **8C**); rear elevation, showing reagent holders (FIG. **8D**); from elevation, showing sample tubes (FIG. **8E**); top, showing insertion of a reagent holder (FIGS. **8F** and **8G**); top showing slot for inserting a reagent holder (FIG. **8H**); top view showing registration of reagent holder (FIG. **8I**); close up of rack in state of partial insertion/removal from apparatus (FIG. **8J**); and rack held by handle, removed from apparatus (FIG. **8K**). A recessed area in a diagnostic or preparatory apparatus, as further described herein, for accepting the exemplary removable rack of FIG. **7** is shown in FIG. **9**. Other suitably configured recessed areas for receiving other racks differing in shape, appearance, and form, rather than function, are consistent with the description herein.

The two exemplary racks shown in the figures being non-limiting, general features of racks contemplated herein are now described using the two exemplary racks as illustrative thereof. For example, the embodiments shown here, at least the first lane and the second lane are parallel to one another, a configuration that increases pipetting efficiency. Typically, when parallel to one another, pairs of adjacent sample lanes are separated by 24 mm at their respective midpoints. (Other distances are possible, such as 18 mm apart, or 27 mm apart. The distance between the midpoints is dependent on the pitch of the nozzles in the liquid dispensing head, as further described herein. Keeping the spacing in multiples of 9 mm enables easy loading from the rack into a **96** well plate (where typically wells are spaced apart by 9 mm). Typically, also, the rack is such that plurality of reagent holders in the plurality of lanes are maintained at the same height relative to one another.

The rack is configured to accept a reagent holder in such a way that the reagent holder snaps or locks reversibly into place, and remains steady while reagents are accessed in it, and while the rack is being carried from one place to another or is being inserted into, or removed from, the apparatus. In each embodiment, each of the second locations comprises a mechanical key configured to accept the reagent holder in a single orientation. In FIG. **3**, it is shown that the reagent holder(s) slides horizontally into vertically oriented slots, one per holder, located in the rack. In such an embodiment, the edge of a connecting member on the holder engages with a complementary groove in the upper portion of a slot. In FIGS. **8F**, **8G**, and **8I**, it is shown that the reagent holder(s) can engage with the rack via a mechanical key that keeps the holders steady and in place. For example, the mechanical key can comprise a raised or recessed portion that, when engaging with a complementary portion of the reagent holder, permits the reagent holder to snap into the second location. It can also be seen in the embodiments shown that the reagent holder has a first end and a second end, and the mechanical key comprises a first feature configured to engage with the first end, and a second feature configured to engage with the second end in such a way that a reagent holder cannot be inserted the wrong way around.

In certain embodiments the reagent holders each lock into place in the rack, such as with a cam locking mechanism that is recognized as locked audibly and/or physically, or such as with a mechanical key. The rack can be configured so that the holders, when positioned in it, are aligned for proper pipette tip pick-up using a liquid dispenser as further described herein. Furthermore, the second location of each lane can be deep enough to accommodate one or more pipette tips, such as contained in a pipette tip sheath.

In certain embodiments, the rack is configured to accept the samples in individual sample tubes **802**, each mounted adjacent to a corresponding holder **804**, for example on one

US 10,632,466 B1

13

side of rack **800**. The sample tubes can be accessible to a sample identification verifier such as a bar code reader, as further described herein. In FIG. **5**, a sample tube is held at its bottom by a cylindrical receiving member. In FIG. **7**, it is shown that a sample tube can be held at both its top and bottom, such as by a recessed portion **803** configured to receive a bottom of a sample tube, and an aperture **805** configured to hold an upper portion of the sample tube. The aperture can be a ring or an open loop, or a hole in a metal sheet. The recessed portion can be as in FIG. **7**, wherein it is an angled sheet of metal housing having a hole large enough to accommodate a sample tube.

The rack can be designed so that it can be easily removed from the apparatus and carried to and from the laboratory environment external to the apparatus, such as a bench, and the apparatus, for example, to permit easy loading of the sample tube(s) and the reagent holder(s) into the rack. In certain embodiments, the rack is designed to be stable on a horizontal surface, and not easily toppled over during carriage, and, to this end, the rack has one or more (such as 2, 3, 4, 6, 8) feet **809**. In certain embodiments, the rack has a handle **806** to ease lifting and moving, and as shown in FIG. **5**, the handle can be locked into a vertical position, during carriage, also to reduce risk of the rack being toppled over. The handle can optionally have a soft grip **808** in its middle. In the embodiment of FIG. **7**, the carrying handle is positioned about an axis displaced from an axis passing through the center of gravity of the rack when loaded, and is free to fall to a position flush with an upper surface of the rack, under its own weight.

The embodiment of FIG. **5** has a metallic base member **810** having 4 feet **811** that also serve as position locators when inserting the rack into the dedicated portion of the apparatus. The handle is attached to the base member. The portion of the rack **812** that accepts the samples and holders can be made of plastic, and comprises 12 slots, and may be disposable.

In the embodiment of FIG. **7**, the rack comprises a housing, a plurality of lanes in the housing, and wherein each lane of the plurality of lanes comprises: a first location configured to accept a sample tube; and a second location, configured to accept a reagent holder; and a registration member complementary to a receiving bay of a diagnostic apparatus. Typically, the housing is made of a metal, such as aluminum, that is both light but also can be machined to high tolerance and is sturdy enough to ensure that the rack remains stable when located in the diagnostic apparatus. The registration member in FIG. **7** comprises four (4) tight tolerance pegs **815**, located one per corner of the rack. Such pegs are such that they fit snugly and tightly into complementary holes in the receiving bay of the apparatus and thereby stabilize the rack. Other embodiments having, for example, 2, or 3, or greater than 4 such pegs are consistent with the embodiments herein.

In particular, the housing in the embodiment of FIG. **7** comprises a horizontal member **821**, and two or more vertical members **822** connected to the horizontal member, and is such that the second location of each respective lane is a recessed portion within the horizontal member. The two or more vertical members **809** in the embodiment of FIG. **7** are configured to permit the rack to free stand thereon. The housing may further comprise two or more feet or runners, attached symmetrically to the first and second vertical members and giving the rack additional stability when positioned on a laboratory bench top.

Furthermore, in the embodiment of FIG. **7**, the housing further comprises a plurality of spacer members **825**, each of

14

which is disposed between a pair of adjacent lanes. Optionally, such spacer members may be disposed vertically between the lanes.

Although not shown in the FIGS., a rack can further comprise a lane identifier associated with each lane. A lane identifier may be a permanent or temporary marking such as a unique number or letter, or can be an RFID, or bar-code, or may be a colored tag unique to a particular lane.

A rack is configured so that it can be easily placed at the appropriate location in the instrument and gives the user positive feedback, such as audibly or physically, that it is placed correctly. In certain embodiments, the rack can be locked into position, it is desirable that the rack be positioned correctly, and not permitted to move thereafter, so that movement of the liquid dispenser will not be compromised during liquid handling operations. The rack therefore has a registration member to ensure proper positioning. In the embodiment of FIG. **7**, the registration member comprises two or more positioning pins configured to ensure that the rack can only be placed in the diagnostic apparatus in a single orientation; and provide stability for the rack when placed in the diagnostic apparatus. The embodiment of FIG. **7** has, optionally, a sensor actuator **817** configured to indicate proper placement of the rack in the diagnostic apparatus. Such a sensor may communicate with a processor **980** to provide the user with a warning, such as an audible warning, or a visual warning communicated via an interface, if the rack is not seated correctly. It may also be configured to prevent a sample preparation process from initiating or continuing if a seating error is detected.

In certain embodiments, the interior of the rack around the location of process tubes in the various holders is configured to have clearance for a heater assembly and/or a magnetic separator as further described herein. For example, the rack is configured so that process chambers on the individual holders are accepted by heater units in a heater assembly as further described herein.

Having a removable rack enables a user to keep a next rack loaded with samples and in line while a previous rack of samples is being prepared by the apparatus, so that the apparatus usage time is maximized.

The rack can also be conveniently cleaned outside of the instrument in case of any sample spills over it or just as a routine maintenance of laboratory wares.

In certain embodiments the racks have one or more disposable parts.

Holder

FIGS. **10A** and **10B** show views of an exemplary holder **501** as further described herein. FIG. **11** shows a plan view of another exemplary holder **502**, as further described herein. FIG. **12A** shows an exemplary holder **503** in perspective view, and FIG. **12B** shows the same holder in cross-sectional view. FIG. **12C** shows an exploded view of the same holder as in FIGS. **12A** and **12B**. All of these exemplary holders, as well as others consistent with the written description herein though not shown as specific embodiments, are now described.

The exemplary holders shown in FIGS. **10A**, **10B**, **11**, **12A**, **12**, and **12C** can each be referred to as a “unitized disposable strip”, or a “unitized strip”, because they are intended to be used as a single unit that is configured to hold all of the reagents and receptacles necessary to perform a sample preparation, and because they are laid out in a strip format. It is consistent with the description herein, though, that other geometric arrangements of the various receptacles

US 10,632,466 B1

15

are contemplated, so that the description is not limited to a linear, or strip, arrangement, but can include a circular or grid arrangement.

Some of the reagents contained in the holder are provided as liquids, and others may be provided as solids. In some embodiments, a different type of container or tube is used to store liquids from those that store the solids.

The holder can be disposable, such as intended for a single use, following which it is discarded.

The holder is typically made of a plastic such as polypropylene. The plastic is such that it has some flexibility to facilitate placement into a rack, as further described herein. The plastic is typically rigid, however, so that the holder will not significantly sag or flex under its own weight and will not easily deform during routine handling and transport, and thus will not permit reagents to leak out from it.

The holder comprises a connecting member **510** having one or more characteristics as follows. Connecting member **510** serves to connect various components of the holder together. Connecting member **510** has an upper side **512** and, opposed to the upper side, an underside **514**. In FIG. **10B**, a view of underside **514** is shown, having various struts **597** connecting a rim of the connecting member with variously the sockets, process tube, and reagent tubes. Struts **597** are optional, and may be omitted all or in part, or may be substituted by, in all or in part, other pieces that keep the holder together.

The holder is configured to comprise: a process tube **520** affixed to the connecting member and having an aperture **522** located in the connecting member; at least one socket **530**, located in the connecting member, the socket configured to accept a disposable pipette tip **580**; two or more reagent tubes **540** disposed on the underside of the connecting member, each of the reagent tubes having an inlet aperture **542** located in the connecting member, and one or more receptacles **550**, located in the connecting member, wherein the one or more receptacles are each configured to receive a complementary container such as a reagent tube (not shown) inserted from the upper side **512** of the connecting member.

The holder is typically such that the connecting member, process tube, and the two or more reagent tubes are made from a single piece, such as a piece of polypropylene.

The holder is also typically such that at least the process tube, and the two or more reagent tubes are translucent.

The one or more receptacles **550** are configured to accept reagent tubes that contain, respectively, sufficient quantities of one or more reagents typically in solid form, such as in lyophilized form, for carrying out extraction of nucleic acid from a sample that is associated with the holder. The receptacles can be all of the same size and shape, or may be of different sizes and shapes from one another. Receptacles **550** are shown as having open bottoms, but are not limited to such topologies, and may be closed other than the inlet **552** in the upper side of connecting member **510**. Preferably the receptacles **550** are configured to accept commonly used containers in the field of laboratory analysts, or containers suitably configured for use with the holder herein. The containers are typically stored separately from the holders to facilitate sample handling, since solid reagents normally require different storage conditions from liquid reagents. In particular many solid reagents may be extremely moisture sensitive.

The snapped-in reagent tubes containing different reagents may be of different colors, or color-coded for easy identification by the user. For example they may be made of different color material, such as tinted plastic, or may have

16

some kind of identifying tag on them, such as a color stripe or dot. They may also have a label printed on the side, and/or may have an identifier such as a barcode on the sealing layer on the top.

The containers **554** received by the receptacles **550** may alternatively be an integrated part of the holder and may be the same type of container as the waste chamber and/or the reagent tube(s), or may be different therefrom. (**0168**) In one embodiment, the containers **554** containing lyophilized reagents, disposed in the receptacles **550** (shown, e.g., in FIGS. **12A** and **12C**), are 0.3 ml tubes that have been further configured to have a star pattern (see FIGS. **13A** and **13B**) on their respective bottom interior surfaces. This is so that when a fluid has been added to the lyophilized reagents (which are dry in the initial package), a pipette tip can be bottomed out in the tube and still be able to withdraw almost the entire fluid from the tube, as shown in FIG. **14**, during the process of nucleic acid extraction. The design of the star-pattern is further described elsewhere herein.

The reagent tubes, such as containing the lyophilized reagents, can be sealed across these tops by a metal foil, such as an aluminum foil, with no plastic lining layer, as further described herein.

The embodiments **501**, **502**, and **503** are shown configured with a waste chamber **560**, having an inlet aperture **562** in the upper side of the connecting member. Waste chamber **560** is optional and, in embodiments where it is present, is configured to receive spent liquid reagents. In other embodiments, where it is not present, spent liquid reagents can be transferred to and disposed of at a location outside of the holder, such as, for example, a sample tube that contained the original sample whose contents are being analyzed. Waste chamber **560** is shown as part of an assembly comprising additionally two or more reagent tubes **540**. It would be understood that such an arrangement is done for convenience, e.g., of manufacture; other locations of the waste chamber are possible, as are embodiments in which the waste chamber is adjacent a reagent tube, but not connected to it other than via the connecting member.

The holder is typically such that the connecting member, process tube, the two or more reagent tubes, and the waste chamber (if present) are made from a single piece, made from a material such as polypropylene.

The embodiments **501** and **503** are shown having a pipette sheath **570**. This is an optional component of the holders described herein. It may be permanently or removably affixed to connecting member **510**, or may be formed, e.g., moulded, as a part of a single piece assembly for the holder. For example, exploded view of holder **503** in FIG. **12C** shows lug-like attachments **574** on the upper surface of a removable pipette sheath **570** that engage with complementary recessed portions or holes in the underside **514** of connecting member **510**. Other configurations of attachment are possible. Pipette sheath **570** is typically configured to surround the at least one socket and a tip and lower portion of a pipette tip when the pipette tip is stationed in the at least one socket. In some embodiments, the at least one socket comprises four sockets. In some embodiments the at least one socket comprises two, three, five, or six sockets.

Pipette sheath **570** typically is configured to have a bottom **576** and a walled portion **575** disposed between the bottom and the connecting member. Pipette sheath **570** may additionally and optionally have one or more cut-out portions **572** in the wall **578**, or in the bottom **576**. Such cutouts provide ventilation for the pipette tips and also reduce the total amount of material used in manufacture of the holder. Embodiment **503** has a pipette sheath with no such cutouts.

US 10,632,466 B1

17

In embodiment **501**, such a cutout is shown as an isosceles triangle in the upper portion of the sheath, a similar shaped cutout may be found at a corresponding position in the opposite side of the sheath, obscured from view in FIG. **10A**. Other cutouts could have other triangular forms, circular, oval, square, rectangular, or other polygonal or irregular shapes, and be several, such as many, in number. The wall **578** of pipette sheath **570** may also have a mesh or frame like structure having fenestrations or interstices. In embodiments having a pipette sheath, a purpose of the sheath is to catch drips from used pipette tips, and thereby to prevent cross-sample contamination, from use of one holder to another in a similar location, and/or to any supporting rack in which the holder is situated. Typically, then, the bottom **576** is solid and bowl-shaped (concave) so that drips are retained within it. An embodiment such as **502**, having no pipette sheath, could utilize, e.g., a drip tray or a drainage outlet, suitably placed beneath pipette tips located in the one or more sockets, for the same purpose. In addition to catching drips, the pipette tip sheath prevents or inhibits the tips of other reagent holders—such as those that are situated adjacent to the one in question in a rack as further described herein—from touching each other when the tips are picked up and/or dropped off before or after some liquid processing step. Contact between tips in adjacent holders is generally not intended by, for example, an automated dispensing head that controls sample processing on holders in parallel, but the pipette tips being long can easily touch a tip in a nearby strip if the angle when dropping off of the tip deviates slightly from vertical.

The holders of embodiments **501**, **502**, and **503**, all have a connecting member that is configured so that the at least one socket, the one or more receptacles, and the respective apertures of the process tube, and the two or more reagent tubes, are all arranged linearly with respect to one another (i.e., their midpoints lie on the same axis). However, the holders herein are not limited to particular configurations of receptacles, waste chamber, process tube, sockets, and reagent tubes. For example, a holder may be made shorter, if some apertures are staggered with respect to one another and occupy 'off-axis' positions. The various receptacles, etc., also do not need to occupy the same positions with respect to one another as is shown in FIGS. **12A** and **12B**, wherein the process tube is disposed approximately near the middle of the holder, liquid reagents are stored in receptacles mounted on one side of the process tube, and receptacles holding solid reagents are mounted on the other side of the process tube. Thus, in FIGS. **10A**, **10B**, and **11**, the process tube is on one end of the connecting member, and the pipette sheath is at the other end, adjacent to, in an interior position, a waste chamber and two or more reagent tubes. Still other dispositions are possible, such as mounting the process tube on one end of the holder, mounting the process tube adjacent the pipette tips and pipette tip sheath (as further described herein), and mounting the waste tube adjacent the process tube. It would be understood that alternative configurations of the various parts of the holder give rise only to variations of form and can be accommodated within other variations of the apparatus as described, including but not limited to alternative instruction sets for a liquid dispensing pipette head, heater assembly, and magnetic separator, as further described herein.

Process tube **520** can also be a snap-in tube, rather than being part of an integrated piece. Process tube **520** is typically used for various mixing and reacting processes that occur during sample preparation. For example, cell lysis can occur in process tube **520**, as can extraction of nucleic acids.

18

Process tube **520** is then advantageously positioned in a location that minimizes, overall, pipette head moving operations involved with transferring liquids to process tube **520**.

Reagent tubes **540** are typically configured to hold liquid reagents, one per tube. For example, in embodiments **501**, **502**, and **503**, three reagent tubes are shown, containing respectively wash buffer, release buffer, and neutralization buffer, each of which is used in a sample preparation protocol.

Reagent tubes **540** that hold liquids or liquid reagents can be sealed with a laminate structure **598**. The laminate structure typically has a heat seal layer, a plastic layer such as a layer of polypropylene, and a layer of metal such as aluminum foil, wherein the heat seal layer is adjacent the one or more reagent tubes. The additional plastic film that is used in a laminate for receptacles that contain liquid reagents is typically to prevent liquid from contacting the aluminum.

Two embodiments of a laminate structure, differing in their layer structures, are shown in FIG. **15**. In both embodiments, the heat seal layer **602**, for example made of a layer or other such polymer with a low melting point, is at the bottom, adjacent to the top of the holder, when so applied. The plastic layer **604** is typically on top of the heat seal layer, and is typically made of polypropylene, having a thickness in the range 10-50 microns. The metal layer **608** is typically on top of the plastic layer and may be a layer of Al foil bonded to the plastic layer with a layer of adhesive **606**, as in the first embodiment in FIG. **15**, or may be a layer of metal that is evaporated or sputtered into place directly on to the plastic layer. Exemplary thicknesses for the respective layers are shown in FIG. **15**, where it is to be understood that variations of up to a factor of 2 in thickness are consistent with the technology hereto. In particular, the aluminum foil is 0.1-15 microns thick, and the polymer layer is 15-25 microns thick in one embodiment. In another embodiment, the aluminum is 0.1-1 microns thick, and the polymer layer is 25-30 microns thick.

The laminates deployed herein make longer term storage easier because the holder includes the presence of sealed lyophilized reagents as well as liquids sealed in close proximity, which is normally hard to achieve.

In one embodiment, the tops of the reagent tubes have beveled edges so that when an aluminum foil is heat bonded to the top, the plastic melt does not extend beyond the rim of the tube. This is advantageous because, if the plastic melt reduces the inner diameter of the tube, it will cause interference with the pipette tip during operation. In other embodiments, a raised fiat portion **599** facilitates application and removal of laminate **598**. Raised surface **599**, on the upper side of the connecting member, and surrounding the inlet apertures to the reagent tubes and, optionally, the waste chamber, is an optional feature of the holder.

The manner in which liquid is pipetted out is such that a pipette tip piercing through the foil rips through without creating a seal around the pipette tip, as in FIG. **16**. Such a seal around the tip during pipetting would be disadvantageous because a certain amount of air flow is desirable for the pipetting operation. In this instance, a seal is not created because the laminate structure causes the pierced foil to stay in the position initially adopted when it is pierced. The upper five panels in FIG. **16** illustrate the pipetting of a reagent out from a reagent tube sealed with a laminate as further described herein. At A, the pipette tip is positioned approximately centrally above the reagent tube that contains reagent **707**. At B, the pipette tip is lowered, usually controllably lowered, into the reagent tube, and in so doing pierces the

foil **598**. The exploded view of this area shows the edge of the pierced laminate to be in contact with the pipette tip at the widest portion at which it penetrates the reagent tube. At C, the pipette tip is withdrawn slightly, maintaining the tip within the bulk of the reagent **707**. The exploded view shows that the pierced foil has retained the configuration that it adopted when it was pierced and the pipette tip descended to its deepest position within the reagent tube. At D, the pipette tip sucks up reagent **707**, possibly altering its height as more and more older people undergo such tests. At E, the pipette tip is removed entirely from the reagent tube.

The materials of the various tubes and chambers may be configured to have at least an interior surface smoothness and surface coating to reduce binding of DNA and other macromolecules thereto. Binding of DNA is unwanted because of the reduced sensitivity that is likely to result in subsequent detection and analysis of the DNA that is not trapped on the surface of the holder,

The process tube also may have a low binding surface, and allows magnetic heads to slide up and down the inside wall easily without sticking to it. Moreover, it has a hydrophobic surface coating enabling low stiction of fluid and hence low binding of nucleic acids and other molecules.

In some embodiments, the holder comprises a registration member such as a mechanical key. Typically such a key is part of the connecting member **510**. A mechanical key ensures that the holder is accepted by a complementary member in, for example, a supporting rack or a receiving bay of an apparatus that controls pipetting operations on reagents in the holder. A mechanical key is normally a particular-shaped cut-out that matches a corresponding cutout or protrusion in a receiving apparatus. Thus, embodiment **501** has a mechanical key **592** that comprises a pair of rectangular-shaped cut-outs on one end of the connecting member. This feature as shown additionally provides for a tab by which a user may gain a suitable purchase when inserting and removing the holder into a rack or another apparatus. Embodiments **501** and **502** also have a mechanical key **590** at the other end of connecting member **510**. Key **590** is an angled cutout that eases insertion of the holder into a rack, as well as ensures a good registration therein when abutting a complementary angled cut out in a recessed area configured to receive the older. Other variations of a mechanical key are, of course, consistent with the description herein: for example, curved cutouts, or various combinations of notches or protrusions all would facilitate secure registration of the holder.

In some embodiments, not shown in FIGS. **10A**, **10B**, **11**, or **12A-C**, the holder further comprises an identifier affixed to the connecting member. The identifier may be a label, such as a writable label, a bar-code, a 2-dimensional bar-code, or an RFID tag. The identifier can be, e.g., for the purpose of revealing quickly what combination of reagents is present in the holder and, thus, for what type of sample preparation protocol it is intended. The identifier may also indicate the batch from which the holder was made, for quality control or record-keeping purposes. The identifier may also permit a user to match a particular holder with a particular sample.

It should also be considered consistent with the description herein that a holder additionally can be configured to accept a sample, such as in a sample tube. Thus, in embodiments described elsewhere herein, a rack accepts a number of sample tubes and a number of corresponding holders in such a manner that the sample tubes and holders can be separately and independently loaded from one another. Nevertheless, in other embodiments, a holder can be configured

to also accept a sample, for example in a sample tube. And thus, a complementary rack is configured to accept a number of holders, wherein each holder has a sample as well as reagents and other items. In such an embodiment, the holder is configured so that the sample is accessible to a sample identification verifier.

Kits

The holder described herein may be provided in a sealed pouch, to reduce the chance of air and moisture coming into contact with the reagents in the holder. Such a sealed pouch may contain one or more of the holders described herein, such as 2, 4, 6, 8, 10, 12, 16, 20, or 24 holders.

The holder may also be provided as part of a kit for carrying out sample preparation, wherein the kit comprises a first pouch containing one or more of the holders described herein, each of the holders configured with liquid reagents for, e.g., lysis, wash, and release, and a second pouch, having an inert atmosphere inside, and one or more reagent tubes containing lyophilized PCR reagents, as shown in FIG. **17**. Such a kit may also be configured to provide for analysis of multiple samples, and contain sufficient PCR reagents (or other amplification reagents, such as for RT-PCR, transcription mediated amplification, strand displacement amplification, NASBA, helicase dependent amplification, and other familiar to one of ordinary skill in the art, and others described herein) to process such samples, and a number of individual holders such as 2, 4, 6, 8, 10, 12, 16, 20, or 24 holders.

Reagent Tubes

As referenced elsewhere herein, the containers **554** that contain lyophilized reagents are 0.3 ml tubes that have been further configured to have a star-shaped—or stellated—pattern (see FIGS. **13A** and **13B**) on their respective bottom interior surfaces. Still other tubes for use herein, as well as for other uses not herein described, can be similarly configured. Thus, for example, the benefits afforded by the star-shaped pattern also accrue to reagent tubes that contain liquid samples that are directly pipetted out of the tubes (as well as to those tubes that initially hold solids that are constituted into liquid form prior to pipetting). Other size tubes that would benefit from such a star-shaped pattern have sizes in the range 0.1 ml to 0.65 ml for example.

The star-shaped pattern ensures that when a fluid is withdrawn from the tube, a pipette tip can be bottomed out in the tube and still be able to withdraw, the entire, or almost the entire fluid from the tube, as shown in FIG. **14**. This is important because, when working with such small volumes, and when target DNA can be present in very few copies, sample loss due to imperfections of pipetting is to be minimized to every extent possible.

The design of the star shaped pattern is important, especially when using for recovery of DNA/RNA present in very small numbers in the clinical sample. The stellated pattern should enable pipetting of most of the liquid (residual volume < 1 microliter) when used with a pipette bottomed out with the bottom of the tube. Additionally, the stellated pattern should be designed to minimize surface area as well as dead-end grooves that tend to have two undesirable effects—to trap liquid as well as to increase undesirable retention of polynucleotides by adsorption.

FIG. **14** is now described, as follows. FIG. **14** has a number of panels, A-G, each representing, in sequence, a stage in a pipetting operation. At A, a pipette tip **2210**, containing a liquid **2211** (such as a buffer solution), is positioned directly or approximately above the center of reagent tube **2200**. The tube contains a number of lyophilized pellets **2212**, and is sealed by a layer **2214**, such

US 10,632,466 B1

21

as of foil. The foil may be heat-sealed onto the top of the tube. Although a laminate layer, as further described herein, can be placed on the reagent tube, typically a layer of aluminum foil is adequate, where the tube contents are solid, e.g., lyophilized, reagents. In some embodiments, the top of the reagent tube has chamfer edges to reduce expansion of the top rim of the tube during heat sealing of a foil on the top of the tube. The tube may further comprise an identifiable code, such as a 1-D or a 2-D bar-code on the top. Such a code is useful for identifying the composition of the reagents stored within, and/or a batch number for the preparation thereof, and/or an expiry date. The code may be printed on with, for example, an inkjet or transfer printer.

Stellated pattern **2203** on the bottom interior surface of the tube **2200** is shown. At B, the pipette tip is lowered, piercing seal **2214**, and brought into a position above the particles **2212**. At C the liquid **2211** is discharged from the pipette tip onto the particles, dissolving the same, as shown at D. After the particles are fully dissolved, forming a solution **2218**, the pipette tip is lowered to a position where it is in contact with the stellated pattern **2203**. At E, the pipette tip is caused to suck up the solution **2218**, and at F, the tip may optionally discharge the solution back into the tube. Steps B and F may be repeated, as desired, to facilitate dissolution and mixing of the lyophilized components into solution. At step G, after sucking up as much of the solution **2218** as is practicable into the pipette tip, the pipette tip is withdrawn from the tube. Ideally, 100% by volume of the solution **2218** is drawn up into the pipette tip at G. In other embodiments, and depending upon the nature of solution **2218**, at least 99% by volume of the solution is drawn up. In still other embodiments, at least 98%, at least 97%, at least 96%, at least 95%, and at least 90% by volume of the solution is drawn up.

The design of the stellated or star-shaped pattern can be optimized to maximize the flow rate of liquid through the gaps in-between a bottomed out pipette, such as a p1000 pipette, and the star pattern, and is further described in U.S. provisional patent application Ser. No. 60/959,437, filed Jul. 13, 2007, incorporated herein by reference. It would be understood that, although the description herein pertains to pipettes and pipette tips typically used in sample preparation of biological samples, the principles and detailed aspects of the design are as applicable to other types of pipette and pipette tip, and may be so-adapted.

FIG. 13A shows a cross sectional perspective view of a reagent tube **2200** having side wall **2201** and bottom **2202**. Interior surface **2204** of the bottom is visible. A star-shaped cutout **2203** is shown in part, as three apical grooves.

Typically the star-shaped pattern is present as a raised portion on the lower interior surface of the tube. Thus, during manufacture of a reagent tube, such as by injection moulding, an outer portion of the mould is a cavity defining the exterior shape of the tube. An interior shape of the tube is formed by a mould positioned concentrically with the outer portion mould, and having a star-shaped structure milled out of its tip. Thus, when liquid plastic is injected into the space between the two portions of the mould, the star-shape is formed as a raised portion on the bottom interior surface of the tube.

The exemplary star pattern **2203** shown in FIG. 13B in plan view resembles a "ship's wheel" and comprises a center **2209**, a circular ring **2207** centered on center **2209**, and 8 radial segments configured as radial grooves **2205**. Each groove meets the other grooves at center **2209**, and has a radial end, also referred to as an apex or vertex. Star pattern **2203** has 8 grooves, but it would be understood that a star

22

pattern having fewer or a greater number of grooves, such as 3, 4, 6, 10, or 12, would be consistent with the design herein. The number of grooves of the star should be minimum consistent with effective liquid pipetting and also spaced apart enough not to trap the tip of any of the pipette tips to be used in the liquid handling applications.

Center **2209** is typically positioned coincidentally with the geometric center of the bottom of reagent tube **2200**. The tube is typically circular in cross-section, so identifying its center (e.g., at a crossing point of two diameters) is normally straightforward. Center **2209** may be larger than shown in FIG. 13B, such as may be a circular cutout or raised portion that exceeds in diameter of the region formed by the meeting point of grooves **2205**.

Ring **2207** is an optional feature of star-shaped pattern **2203**. Typically ring **2207** is centered about center **2209**, and typically it also has a dimension that corresponds to the lower surface of a pipette tip. Thus, when a pipette tip 'bottoms out' in the bottom of reagent tube **2200**, the bottom of the pipette tip rests in contact with ring **2207**. Ring **2207** is thus preferably a cut-out or recessed feature that can accommodate the pipette tip and assist in guiding its positioning centrally at the bottom of the tube. In other embodiments more than one, such as 2, 3, or 4 concentric rings **2207** are present.

The star pattern is configured to have dimensions that give an optimal flow-rate of liquid out of the reagent tube into a suitably positioned pipette tip. The star pattern is shown in FIG. 13B as being significantly smaller in diameter than the diameter of the tube at its widest point. The star pattern may have, in various embodiments, a diameter (measured from center **2209** to apex of a groove **2205**) from 5-20% of the diameter of the reagent tube, or from 10-25% of the diameter of the reagent tube, or from 15-30% of the diameter of the reagent tube, or from 20-40% of the diameter of the reagent tube, or from 25-50% of the diameter of the reagent tube, or from 30-50% the diameter of the reagent tube, or from 40-60% the diameter of the reagent tube, or from 50-75% the diameter of the reagent tube, or from 65-90% the diameter of the reagent tube.

The grooves **2205** are thus separated by ridges (occupying the space in between adjacent grooves). In the embodiment shown, the grooves are narrower (occupy a smaller radial angle) than the gaps between them. In other embodiments, the grooves may be proportionately wider than the gaps between them. In such embodiments, it may be more appropriate to describe them as having ridges instead of grooves, in other embodiments, the grooves and ridges that separate them are of equal widths at each radial distance from the center.

The grooves that form the apices of the star may be rounded in their lower surfaces, such as semi-circular in cross section, but are typically V-shaped. They may also be trapezoid in cross-section, such as having a wider upper portion than the bottom, which is flat, the upper portion and the bottom being connected by sloping walls.

In some embodiments, for ease of manufacture, the grooves end on the same level in the bottom of the tube. Thus the radial ends are all disposed on the circumference of a circle. In other embodiments, the grooves do not all end on the same level. For example, grooves may alternately end on different levels, and thus the ends are alternately disposed on the respective circumferences of two circles that occupy different planes in space from one another.

Grooves **2205** are shown in FIG. 13B as having equal lengths (as measured from center **2209** to apex). This need not be so. In alternative embodiments, grooves may have

different lengths from one another, for example, as alternating lengths on alternating grooves, where there are an even number of grooves. Furthermore, apices may be rounded, rather than pointed.

Typically the grooves taper uniformly in width and depth from center **2209** to each respective apex. Still other configurations are possible, such as a groove that follows a constant width, or depth, out to a particular radial extent, such as 30-60% of its length, and then narrows or becomes shallower towards its apex. Alternatively, a groove may start narrow at center **2209**, widen to a widest region near its midpoint of length, and then narrow towards its apex. Still other possibilities, not described herein, are consistent with the stellated pattern.

In a 0.3 ml tube, the width of each groove **2205** at its widest point is typically around 50 microns, and the width typically tapers uniformly from a widest point, closest to or at center **2209**, to the apex.

In a 0.3 ml tube, the depth of a groove at the deepest point is typically around 25-50 microns and the depth typically tapers uniformly from a deepest point, closest to or at center **2209**, to an apex.

In a 0.3 ml tube, the radius of the star formed from the grooves, measured as the shortest distance from center **2209** to apex, is typically around 0.5 mm. but may be from 0.1-1 mm, or from 0.3-2 mm.

In another embodiment, in a 0.3 ml tube, the grooves should be rounded off and less than 100 microns deep, or less than 50 microns deep, or less than 25 microns deep.

The stellated pattern typically has a rotation axis of symmetry, the axis disposed perpendicular to the bottom of the tube and through center **2209**, so that the grooves are disposed symmetrically about the rotation axis. By this is meant that, for n grooves, a rotation of $2\pi/n$ about the central (rotational) axis can bring each groove into coincidence with the groove adjacent to it.

The stellated shape shown in FIG. 13B is not limiting in that it comprises a number of radially disposed grooves **2205**, and an optional circular ring **2207**. Other star-shaped geometries may be used, and, depending upon ease of manufacture, may be preferred. For example, a star can be created simply by superimposing two or more polygons having a common center, but offset rotationally with respect to one another about the central axis. (See, for example "star polygons" described at the Internet site mathworld.wolfram.com/StarPolygon.html.) Such alternative manners of creating star-shaped patterns are utilized herein.

Liquid Dispenser

In various embodiments, preparation of a PCR-ready sample for use in subsequent diagnosis using the apparatus as further described herein, can include one or more of the following steps: contacting a neutralized polynucleotide sample with a PCR reagent mixture comprising a polymerase enzyme and a plurality of nucleotides (in some embodiments, the PCR reagent mixture can further include a positive control plasmid and a fluorogenic hybridization probe selective for at least a portion of the plasmid); in some embodiments, the PCR reagent mixture can be in the form of one or more lyophilized pellets, as stored in a receptacle on a holder, and the method can further include reconstituting the PCR pellet with liquid to create a PCR reagent mixture solution. Various, such as one or more, of the liquid transfer operations associated with the foregoing steps can be accomplished by an automated pipette head.

A suitable liquid dispenser for use with the apparatus herein comprises one or more sensors; a manifold, one or more pumps in fluid communication with the manifold; one

or more dispense heads in fluid communication with the manifold; and electrical connections that accept electrical signals from an external controller, wherein the liquid dispenser has no inlet or outlet for fluids, other than through the one or more pumps.

A cross-sectional view of an exemplary liquid dispenser is shown in FIG. 18. The liquid dispenser is configured to carry out fluid transfer operations on two or more holders simultaneously. As shown in FIG. 18, liquid dispenser **2105** can be mounted on a gantry having three degrees of translational freedom. Further embodiments can comprise a gantry having fewer than three degrees of translational freedom. The manner of mounting can be by a mechanical fastening such as one or more screws, as shown on the left hand side of FIG. 18. A suitable gantry comprises three axes of belt-driven slides actuated by encoded stepper motors. The gantry slides can be mounted on a framework of structural angle aluminum or other equivalent material, particularly a metal or metal alloy. Slides aligned in x- and y-directions (directed out of and in the plane of FIG. 18 respectively) facilitate motion of the gantry across an array of holders, and in a direction along a given holder, respectively.

The z-axis of the gantry can be associated with a variable force sensor which can be configured to control the extent of vertical motion of the head during tip pick-up and fluid dispensing operations. Shown in FIG. 15, for example, a pipette head **1803** can be mounted such that a force acting upwardly against the head can be sensed through a relative motion between the head and a force sensor. For example, when pipette head **1803** forces against a disposable pipette in the rack below it, an upward force is transmitted causing head **1803** to torque around pivot point **2102**, causing set screw **2104** to press against a force sensor, in turn, the force sensor is in communication with a processor or controller dial controls at least the vertical motion of the liquid dispenser so that, thereby, the processor or controller can send instructions to arrest the vertical motion of the liquid dispenser upon receiving an appropriate signal from the force sensor. An exemplary force sensor suitable for use herein is available from Honeywell; its specification is shown in an appendix hereto. The force sensor mechanism shown in FIG. 18 is exemplary and one of many possible mechanisms capable of commanding the head during up pick-up and fluid dispensing operations. For example, as an alternative to a force sensor, a stall sensor that senses interruption in vertical motion of the one or more dispense heads upon contact with a sample tube or reagent holder may be used. Accordingly, as would be understood by one of ordinary skill in the art, the liquid dispenser as described herein is not limited to the specific mechanism shown in FIG. 18.

The liquid dispenser further comprises a number of individually sprung heads **1803**, wherein each head is configured to accept a pipette tip from the one or more pipette tips in a holder. The liquid dispenser can be further configured such that no two heads accept pipette tips from the same holder. FIGS. 19A-C, for example, depicts four individually sprung heads **1803**, but it is to be understood that the dispenser is not limited to this number. For example, other numbers include 2,3,5,6,8,10, or 12. Furthermore, the individually sprung heads **1803** are shown arranged in parallel to one another, but may be configured in other arrangements.

The liquid dispenser can further comprise computer-controlled pump **2100** connected to distribution manifold **1802** with related computer controlled valving. Distribution manifold **1802** can comprise a number of valves, such as solenoid valves **1801** configured to control the flow of air

US 10,632,466 B1

25

through the pipette tips; in an exemplary embodiment, there are two valves for each pipette, and one additional valve to vent the pump. Thus, for a liquid dispenser having four pipette heads, there are nine valves. In another embodiment there is only one valve for each pipette, and one additional

valve to vent the pump. However, the distribution manifold is not limited so comprising exactly nine solenoid valves. The liquid dispenser is further configured to aspirate or dispense fluid in connection with analysis or preparation of solutions of two or more samples. The liquid dispense is also

configured to dispense liquid into a microfluidic cartridge. Additionally, the liquid dispenser is configured to accept or dispense, in a single operation, an amount of 1.0 ml of fluid or less, such as an amount of fluid in the range 10 nl-1 ml. The liquid dispenser is configured such that pump **2100** pumps air in and out of the distribution manifold. The distribution manifold comprises a microfluidic network that distributes air evenly amongst the one or more valves. Thus, by controlling flow of air through the manifold and various valves, pressure above the pipette heads can be varied so that liquid is drawn up into or expelled from a pipette tip attached to the respective pipette heads. In this way it is not necessary to supply compressed air via an air hose to the liquid dispenser. Neither is it necessary to provide liquid lines to the dispense head. Furthermore, no liquid reagents or liquid samples from the holders enters any part of the liquid dispenser, including the manifold. This aspect reduces complications from introducing air bubbles into samples or liquid reagents. An exemplary configuration of a distribution manifold is shown in FIG. **20**.

As shown in the various figures, the entire liquid dispenser that moves up and down the z-axis is a self-contained unit having only electrical connections to a processor or controller, and mechanical connections to the gantry. The translational motions in three dimensions of the liquid dispenser can be controlled by a microprocessor, such as processor **950**. No fluid handling lines are associated with the dispenser. This design enables simplification of assembly of the instrument, minimizes contamination of the instrument and cross-contamination of samples between different instances of operation of the apparatus, increases efficiency of pumping (minimal dead volume) and enables easy maintenance and repair of the device. This arrangement also enables easy upgrading of features in the dispensing device, such as individual and independent pump control for each dispenser, individual pipette attachment or removal, ability to control the pitch of the pipettes, etc.

Another aspect of the apparatus relates to a sample identification verifier configured to check the identity of each of the number of nucleic-acid containing samples. Such sample identification verifiers can be optical character readers, bar code readers, or radio frequency tag readers, or other suitable readers, as available to one of ordinary skill in the art. A sample identification verifier can be mounted on the gantry, or attached to the liquid dispenser so that it moves in concert with the liquid dispenser. Alternatively, the sample identification verifier can be separately mounted and can move independently of the liquid dispenser. In FIGS. **21** and **22**, for example, sample identification verifier **1701** is a bar-code reader attached to the liquid dispenser. The field of view of barcode scanner **1701** is non-linear, enabling it to detect light reflected by mirror **2300** from the barcoded clinical sample tube **2301** in disposable rack **2302**. The barcode scanner reads the barcode on the clinical sample tube thus identifying the presence and specifics of the sample tube. Because of use of a mirror, the scanner is configured either to read a bar-code printed in mirror image

26

form (that is thus reflected into normal form), or to read a mirror image of a normal bar-code and to convert the mirror image to unreflected form via a computer algorithm.

Sample identification verifier is configured to communicate details of labels that it has detected or read to a processor or controller in the apparatus, thereby permitting sample identifying information to be associated with diagnostic results and other information relating to sample preparation, and extraction and amplification of nucleic acid therein.

In FIG. **23**, the sample identification verifier is positioned to read indicia from a microfluidic cartridge.

In certain embodiments, the liquid dispenser can also comprise one or more sensors **2001** (e.g., infra-red sensors) each of which detects the presence of a pipette tip in a rack. In FIG. **24**, for example, an infra-red sensor **2001** can have an infra-red emitter placed opposed to it, and the presence of disposable pipette tip **2000** obstructs the line of sight between the emitter and the detector, thus enabling determination of the presence or absence of the pipette tip. The disposal pipettes are configured perpendicular to pipette stripper-alignment plate **2003** as further described herein.

The liquid dispenser can also operate in conjunction with a motorized plate configured to strip the pipettes and align the pipettes during dispensing of fluid into a micro fluidic cartridge, as further described herein.

FIGS. **25A** and **25B** show an exemplary device for stripping pipette tips from a liquid dispenser as further described herein. The pipette tips are aligned, all at the same pitch, above respective sockets (over a pipette tip sheath) in a holder. A metal plate having elongated holes lies over the sockets. The pipette tips are inserted part way down into the sheath through the elongated holes, and the metal plate is moved along in such a manner that the pipette tips are clamped by the elongated portion of the holes. When the liquid dispenser is moved up, the pipette tips become detached from their respective heads. When the metal plate is subsequently moved back to its initial position, the pipette tips remain in place in their respective sockets.

Heater Assembly & Magnetic Separator

A cross-sectional view of a heater unit of an exemplary heater assembly **1401** is shown in FIG. **18** (right hand panel). The heater assembly comprises one or more independently controllable heater units, each of which comprises a heat block. In certain embodiments there are 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24, 25, 30, 32, 36, 40, 48, or 50 heater units in a heater assembly. Still other numbers of heater units, such as any number between 6 and 100 are consistent with the description herein. The one or more heat blocks may be fashioned from a single piece of metal or other material, or may be made separately from one another and mounted independently of one another or connected to one another in some way. Thus, the term heater assembly connotes a collection of heater units but does not require the heater units or their respective heat blocks to be attached directly or indirectly to one another. The heater assembly can be configured so that each heater unit independently heats each of the one or more process tubes **1402**, for example by permitting each of the one or more heat blocks to be independently controllable, as further described herein. In the configuration of FIG. **26**, the heater assembly comprises one or more heat blocks **1403** each of which is configured to align with and to deliver heat to a process tube **1402**. Each heat block **1403** can be optionally secured and connected to the rest of the apparatus using a strip **1408** and one or more screws **1407** or other adhesive device. This securing mechanism is not limited to such a configuration.

US 10,632,466 B1

27

Although a cross-sectional view of one heat block **1403** is shown in FIG. **26**, it should be understood that this is consistent with having multiple heat blocks aligned in parallel to one another and such that their geometric mid-points all be on a single linear axis, though it is not so limited in configuration. Thus, the one or more heat blocks may be positioned at different heights from one another, in groups or, alternately, individually, or may be staggered with respect to one another from left to right in FIG. **26** (right hand panel), in groups or alternately, or individually. Additionally, and in other embodiments, the heat blocks are not aligned parallel to one another but are disposed at angles relative to one another, the angles being other than 180°. Furthermore, although the heat block shown in FIG. **26** may be one of several that are identical in size, it is consistent with the technology herein that one or more heat blocks may be configured to accept and to heat process tubes of different sizes.

The exemplary heat block **1403** in FIG. **26** (right hand panel) is configured to have an internal cavity that partially surrounds a lower portion of process tube **1402**. In the heat block of FIG. **26**, the internal cavity surrounds the lower portion of process tube **1402** on two sides but not the front side (facing away from magnet **1404**) and not the rear side (adjacent to magnet **1404**). In other embodiments, heat block **1403** is configured to surround the bottom of process tube **1402** on three sides, including the front side. Still other configurations of heat block **1403** are possible, consistent with the goals of achieving rapid and uniform heating of the contents of process tube **1402**. In certain embodiments, the heat block is shaped to conform closely to the shape of process tube **1402** so as to increase the surface area of the heat block that is in contact with the process tube during heating of the process tube. Thus, although exemplary heat block **1403** is shown having a conical, curve-bottomed cavity in which a complementary process tube is seated, other embodiments of heat block **1403** have, for example, a cylindrical cavity with a flat bottom. Still other embodiments of heat block **1403** may have a rectilinear internal cavity such as would accommodate a cuvette.

Moreover, although heat block **1403** is shown as an L-shape in FIG. **26**, which aids in the transmittal of heat from heating element **1501** and in securing the one or more heat blocks to the rest of the apparatus, it need not be so, as further described herein. For example, in some embodiments heating element **1501** may be positioned directly underneath process tube **1402**.

Each heat block **1403** is configured to have a low thermal mass while still maintaining high structural integrity and allowing a magnet to slide past the heat blocks and the process tubes with ease. A low thermal mass is advantageous because it allows heat to be delivered or dissipated rapidly, thus increasing the heating and cooling efficiency of the apparatus in which the heater assembly is situated. Factors that contribute to a low thermal mass include the material from which a heat block is made, and the shape that it adopts. The heat blocks **1403** can therefore be made of such materials as aluminum, silver, gold, and copper, and alloys thereof, but are not so limited.

In one embodiment, the heat block **1403** has a mass of ~10 grams and is configured to heat up liquid samples having volumes between 1.2 ml and 10 µl. Heating from room temperature to 65° C. for a 1 ml biological sample can be achieved in less than 3 minutes, and 10 µl of an aqueous liquid such as a release buffer up to 85° C. (from 50° C.) in less than 2 minutes. The heat block **1403** can cool down to 50° C. from 85° C. in less than 3 minutes. The heat block

28

1403 can be configured to have a temperature uniformity of 65±4° C. for heating up 1 ml of sample and 85±3° C. for heating up 10 µl of release buffer. These ranges are typical, but the heat block can be suitably scaled to heat other volumes of liquid at rates that are slower and faster than those described. This aspect of the technology is one aspect that contributes to achieving rapid nucleic acid extraction of multiple samples by combination of liquid processing steps, rapid heating for lysis, DNA capture and release and magnetic separation, as further described herein.

Not shown in FIG. **26**, the heater assembly **1401** can also optionally be contained in an enclosure that surrounds the heat blocks **1403**. The enclosure can be configured to enable sufficient air flow around the process tubes and so as not to significantly inhibit rate of cooling. The enclosure can have a gap between it and the heat blocks to facilitate cooling. The enclosure can be made of plastic, but is not so limited. The enclosure is typically configured to appear aesthetic to a user.

As shown in FIG. **26**, the heater assembly **1401** can also comprise one or more heating elements (e.g., a power resistor) **1501** each of which is configured to thermally interface to a heat block **1403** and dissipate heat to it. For example, in one embodiment, a power resistor can dissipate up to 25 Watts of power. A power resistor is advantageous because it is typically a low-cost alternative to a heating element. Other off-the-shelf electronic components such as power transistors may also be used to both sense temperature and heat. Although the heating element **1501** is shown placed at the bottom of the heat block **1403**, it would be understood that other configurations are consistent with the assembly described herein, for example, the heating element **1501** might be placed at the top or side of each heat block **1403**, or directly underneath process tube **1402**. In other embodiments, the heating element has other shapes and is not rectangular in cross section but may be curved, such as spherical or ellipsoidal. Additionally, the heating element may be moulded or shaped so that it conforms closely or approximately to the shape of the bottom of the process tube. Not shown in FIG. **26**, the heater assembly can also comprise an interface material (e.g., Berquist q-pad, or thermal grease) between the heating element **1501** and the heat block **1403** to enable good thermal contact between the element and the heat block.

In the embodiment shown in FIG. **26**, the heater assembly further comprises one or more temperature sensors **1502**, such as resistive temperature detectors, to sense the respective temperatures of each heat block **1403**. Although a temperature sensor **1502** is shown placed at the bottom of the heat block **1403**, it would be understood that other configurations are consistent with the assembly described herein: for example, the temperature sensor might be placed at the top or side of each heat block **1403**, or closer to the bottom of process tube **1402** but not so close as to impede uniform heating thereof. As shown in the embodiment of FIG. **26**, the heater assembly can further comprise an interface material (e.g., Berquist q-pad) **1503** configured to enable good thermal contact between the sensor **1502** and the heat block **1403**, to thereby ensure an accurate reading.

Certain embodiments of the diagnostic or preparatory apparatus herein have more than one heater assembly as further described herein. For example, a single heater assembly may be configured to independently heat 6 or 12 process tubes, and an apparatus may be configured with two or four such heater assemblies.

The disclosure herein further comprises a magnetic separator, configured to separate magnetic particles, the separa-

US 10,632,466 B1

29

tor comprising: one or more magnets affixed to a supporting member; a motorized mechanism configured to move the supporting member in such a manner that the one or more magnets move backwards and forwards along a fixed axis, and during at least a portion of the motion, the one or more magnets maintain close proximity to one or more receptacles which contain the magnetic particles in solution; and control circuitry to control the motorized mechanism.

The disclosure herein still further includes an integrated magnetic separator and heater, comprising: a heater assembly, wherein the heater assembly comprises a plurality of independently controllable heater units, each of which is configured to accept and to heat one of a plurality of process tubes; one or more magnets affixed to a supporting member: a motorized mechanism configured to move the supporting member in such a manner that the one or more magnets move backwards and forwards along a fixed axis, and during at least a portion of the motion the one or more magnets maintain close proximity to one or more of the process tubes in the heater assembly, wherein the one or more process tubes contain magnetic particles; and control circuitry to control the motorized mechanism and to control heating of the heater units.

Typically, each of the one or more receptacles is a process tube, such as for carrying out biological reactions. In some embodiments, close proximity can be defined as a magnet having a face less than 2 mm away from the exterior surface of a process tube without being in contact with the tube. It can still further be defined to be less than 1 mm away without being in contact with the tube, or between 1 and 2 mm away.

Typically the magnetic particles are microparticles, heads, or microspheres capable of binding one or more biomolecules, such as polynucleotides. Separating the particles, while in solution, typically comprises collecting and concentrating, or gathering, the particles into one location in the inside of the one or more receptacles.

An exemplary magnetic separator **1400** is shown in FIG. **27**, configured to operate in conjunction with heater assembly **1401**. The magnetic separator **1400** is configured to move one or more magnets relative to the one or more process tubes **1402**. While the magnet **1404** shown in FIG. **27** is shown as a rectangular block, it is not so limited in shape. Moreover, the configuration of FIG. **27** is consistent with either having a single magnet that extends across all heat blocks **1403** or having multiple magnets operating in concert and aligned to span a subset of the heat blocks, for example, aligned collinearly on the supporting member. The magnet **1404** can be made of neodymium (e.g., from K & J Magnetics, Inc.) and can have a magnetic strength of 5,000-15,000 Gauss (Brmax). The poles of the magnets **1404** can be arranged such that one pole faces the heat blocks **1403** and the other faces away from the heat blocks.

Further, in the embodiment shown in FIG. **27**, the magnet **1404** is mounted on a supporting member **1505** that can be raised up and down along a fixed axis using a motorized shaft **1405**. The fixed axis can be vertical. In the embodiment shown in FIG. **27**, a geared arrangement **1406** enables the motor **1601** to be placed perpendicular to the shaft **1405**, thereby saving space in the apparatus in which magnetic separator **1400** is situated. In other embodiments, the motor is placed underneath shaft **1405**. It would be understood that other configurations are consistent with the movement of the magnet relative to the process tubes, including, but not limited to, moving the magnet from side-to-side, or bringing the magnet down from above. The motor can be computer controlled to run at a particular speed; for example at a

30

rotational speed that leads to vertical motion of the magnet in the range 1-20 mm/s. The magnetic separator can thus be configured to move repetitively, e.g., up and down, from side to side, or backwards and forwards, along the same axis several times. In some embodiments there is more than one shaft that operates under motorized control. The presence of at least a second shaft has the effect of making the motion of the separator more smooth. In some embodiments, the supporting member rides on one or more guiding members to ensure that the supporting member does not, for example, tip, twist, or yaw, or undergo other internal motions while moving (other than that of controlled motion along the axis) and thereby reduce efficacy of the separation.

The supporting member can also be configured to move the magnets between a first position, situated away from the one or more receptacles, and a second position situated in close proximity to the one or more receptacles, and is further configured to move at an amplitude about the second position where the amplitude is smaller than a distance between the first position and the second position as measured along the shaft.

Shown in FIGS. **26** and **27**, the heater assembly **1401** and the magnetic separator **1400** can be controlled by electronic circuitry such as on printed circuit board **1409**. The electronic circuitry **1409** can be configured to cause the heater assembly **1401** to apply heat independently to the process tubes **1402** to minimize the cost of heating and sensing. It can also be configured to cause the magnetic separator **1400** to move repetitively relative to the process tubes **1402**. The electronic circuitry **1409** can be integrated into a single printed circuit board (PCB). During assembly, a plastic guide piece can help maintain certain spacing between individual heat blocks **1403**. This design can benefit from use off-the-shelf electronics to control a custom arrangement of heat blocks **1403**.

Not shown in FIGS. **26** and **21**, an enclosure can cover the magnetic separator **1400** and the heater assembly **1401** for protection of sub-assemblies below and aesthetics. The enclosure can also be designed to keep the heat blocks **1403** spaced apart from one another to ensure efficiency of heating and cooling. The magnetic separator and heater assembly can, alternatively, be enclosed by separate enclosures. The one or more enclosures can be made of plastic.

Advantageously, the heater assembly and magnetic separator operate together to permit successive heating and separation operations to be performed on liquid materials in the one or more process tubes without transporting either the liquid materials or the process tubes to different locations to perform either heating or separation. Such operation is also advantageous because it means that the functions of heating and separation which, although independent of one another, are both utilized in sample preparation may be performed with a compact and efficient apparatus.

Cartridge Autoloader

An exemplary embodiment of a PCR amplification-detection system **2900** for use with a microfluidic cartridge is shown in FIG. **28**. The system **2900** performs and automates the process of PCR on multiple nucleic-acid containing samples in parallel. The system **2900** comprises a depository **2907** for unused micro fluidic cartridges, a cartridge autoloader, a receiving bay for a microfluidic cartridge, a detector, and a waste tray **2903** configured to receive used microfluidic cartridges. In one embodiment, the cartridge autoloader comprises a cartridge pack **2901**, and a cartridge pusher **2904**.

The system **2900**, for illustration purposes, is configured so that a microfluidic cartridge moves in a plane and in a

US 10,632,466 B1

31

linear manner from the depository to the receiving bay, to the waste bin, but it need not be so arranged. For example, the waste cartridge bin **2903** can be aligned orthogonally, or any angle thereof, to the receiving bay, such as disposed behind it. Alternatively, each element (cartridge autoloader **2901**, receiving bay **2902**, and waste cartridge bin **2903**) can be configured in a step-wise manner where the cartridge pack **2901** is on the same, higher or lower level than the microfluidic PCR amplification-detection system **2902** and the microfluidic PCR amplification-detection system **2902** is on the same, higher or lower level than the waste cartridge bin **2903**. Another configuration could be that each of the three elements is not arranged linearly but at an angle to one another, although within the same plane.

FIG. **28** illustrates the cartridge pack **2901** and the waste cartridge bin **2903** below the plane of the receiving bay, and a detection system **2908** above the plane. This configuration is exemplary and it would be understood that these elements may be positioned above or below the plane in other embodiments.

FIG. **29** illustrates a depository for unused microfluidic cartridges. The depository can be configured to accept a number of individually stacked and individually loaded cartridges, or can be configured to accept a pack of cartridges. An exemplary cartridge pack has 24 cartridges. The depository may consist of a cage **2910** of any material that may or may not be transparent. For example it may be made of metal or plastic. The cartridge pack **2901** is not limited to twenty-four cartridges 106 per pack but may contain any number from 2 to 100. For example, other numbers such as 2, 4, 8, 10, 12, 16, 20, 30, 36, 40, 48, 50, or 64 are possible numbers of cartridges 106 per pack. Similarly, the depository may be configured to accept those numbers of cartridges, when individually stacked. In one embodiment, as in FIG. **29**, each cartridge **2906**, individually stacked, rests on ledges **2911** that protrude from the cage **2910**. However, other configurations are possible. For example, a cartridge **2906** may rest on recessed grooves made within the interior surfaces of cage **2910**. Furthermore, the cartridge pack **2901** may not need to be placed in a cage **2910**. The cartridge pack **2901** may itself include the necessary connections to bind securely to the apparatus to load the cartridges **2906**.

FIG. **30** is an illustration of an exemplary initial loading position of a cartridge pack **2901** in a depository when samples are loaded in the topmost cartridge in the pack. FIG. **30** shows the cartridge pack **2901** below a plane that contains a cartridge pusher. In other embodiments, the cartridge pack **2901** may be above the plane of a cartridge pusher where the pusher pushes the lowest cartridge out from the holder; or partly above and partly below in a holder **2920** where a cartridge pusher pushes a cartridge from the middle of the cartridge pack **2901**. In the embodiment shown, a topmost cartridge **106** is pushed along two guide rails **2905**. Alternatively, there may be more or fewer guide rails (such as one or three) or no guide rails at all so long as a cartridge **2906** can be caused to move to other required positions.

An exemplary cartridge pusher **2904** is shown in FIG. **31**. The cartridge pusher **2904** pushes a cartridge **2906** along guide rails **2905**, which allows a cartridge **2906** to travel to pre-calibrated positions by the mechanism of a stepper motor **2930**. However, it would be understood that the mechanism of transporting the cartridge **2906** is not limited to a stepper motor **2930** and thus other mechanisms are also consistent with the cartridge pusher **2904** as described herein.

32

FIG. **32** shows a used cartridge **2906** that has been pushed by the cartridge pusher **2904** into the waste cartridge bin **2903** after a PCR process has been completed. The embodiment shows a lipped handle **2940** that facilitates easy handling, such as emptying, of the bin **2903**. However, it would be understood that the handle **2904** is not limited to the style and shape shown.

An exemplary cartridge pack **2901**, before and after multiple PCR processes are completed are shown in FIG. **33**. After the cartridge pusher **2904** pushes a cartridge **2906** out of the cartridge pack **2901**, a spring **2950** at the bottom of the cartridge pack pushes against the lower surface of the stack of cartridges and causes the topmost cartridge to be made available for sample injection. The spring **2950** is not limited in number or type. Thus although a single helical or coiled spring is shown, it is consistent with the description herein that more than one helical or coiled springs could be used, such as 2, 3, or 4, and that alternatively a sprung metal strip, or several strips, could be used. Alternatively another mechanism for forcing the cartridges upwards could be deployed, such as a pneumatic, hydraulic, or inflatable pressurized container, could be utilized.

It is to be noted that microfluidic cartridges, as further described herein, that have a raised lip along their edges to permit ease of stacking and/or storage in a pack or an autoloader are particularly advantageous because the raised lips also introduce a stiffness into the cartridges and assist in keeping the fluid inlets on one cartridge away from those on another cartridge during storage and transport. The raised regions, which need not only be lips along each edge of a cartridge, also help minimize friction between the lower surface of one cartridge and the upper surface of another. Cartridge Receiving Bay

The present technology relates to an apparatus and related methods for amplifying, and carrying out diagnostic analyses on, nucleotides from biological samples. The apparatus is configured to act on a disposable microfluidic cartridge containing multiple sample lanes in parallel, and comprises a reusable instrument platform that can actuate un-cartridge operations, can detect and analyze the products of the PCR amplification in each of the lanes separately, in all simultaneously, or in groups simultaneously, and, optionally, can display the results on a graphical user interface.

FIG. **34** shows a perspective view of an exemplary cartridge **200** that contains multiple sample lanes, and exemplary read head **300** that contains detection apparatus for reading signals from cartridge **200**. Also shown in FIG. **34** is a tray **110** that, optionally, can accommodate cartridge **200** prior to insertion of the cartridge in a receiving bay. The apparatus described herein is able to carry out real-time PCR on a number of samples in cartridge **200** simultaneously. Preferably the number of samples is 12 samples, as illustrated with exemplary cartridge **200**, though other numbers of samples such as 4, 8, 10, 16, 20, 24, 25, 30, 32, 36, 40, and **48** are within the scope of the present description. In preferred operation of the apparatus, a PCR-ready solution containing the sample, and, optionally, one or more analyte-specific reagents (ASR's) using other components of the apparatus, as further described herein, prior to introduction into cartridge **200**.

In some embodiments, an apparatus includes a bay configured to selectively receive a microfluidic cartridge; at least one heat source thermally coupled to the bay; and coupled to a processor as further described herein, wherein the heat source is configured to heat individual sample lanes in the cartridge, and the processor is configured to control

application of heat to the individual sample lanes, separately, in all simultaneously, or in groups simultaneously.

In some embodiments, an apparatus further includes at least one detector configured to detect a polynucleotide (nucleic acid) in a sample in one or more of the individual sample lanes, separately or simultaneously; wherein the processor is coupled to the detector to control the detector and to receive signals from the detector.

The bay can be a portion of the apparatus that is configured to selectively receive the microfluidic cartridge. For example, the bay and the microfluidic cartridge can be complementary in shape so that the microfluidic cartridge is selectively received in, e.g., a single orientation. For example, the microfluidic cartridge can have a registration member that fits into a complementary feature of the bay. The registration member can be, for example, a cut-out on an edge of the cartridge, such as a corner that is cut-off, or one or more notches that are made on one or more of the sides. By selectively receiving the cartridge, the bay can help a user to place the cartridge so that the apparatus can properly operate on the cartridge. In this way, error-free alignment of cartridges can be achieved. Moreover, the cartridge can be designed to be slightly smaller than the receiving bay by approximately 200-300 micron for easy placement and removal of the cartridge. The apparatus can further include a sensor configured to sense whether the microfluidic cartridge is selectively received.

The bay can also be configured so that various components of the apparatus that can operate on the microfluidic cartridge (heat sources, detectors, force members, and the like) are positioned to properly operate on the microfluidic cartridge. For example, a contact heat source can be positioned in the bay such that it can be thermally coupled to a distinct location at a microfluidic cartridge that is selectively received in the receiving bay.

Alternatively, in connection with alignment of microheaters in the heater module with corresponding heat-requiring microcomponents (such as valves, pumps, gates, reaction chambers, etc.), the microheaters can be designed to be slightly bigger than the heat requiring microfluidic components so that even though the cartridge may be off-centered from the heater, the individual components can still function effectively.

The detector **300** can be, for example, an optical detector, as further described herein. For example, the detector can include a light source that selectively emits light in an absorption band of a fluorescent dye, and a light detector that selectively detects light in an emission band of the fluorescent dye, wherein the fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof. Alternatively, for example, the optical detector can include a bandpass-filtered diode that selectively emits light in the absorption band of the fluorescent dye and a bandpass filtered photodiode that selectively detects light in the emission band of the fluorescent dye; or for example, the optical detector can be configured to independently detect a plurality of fluorescent dyes having different fluorescent emission spectra, wherein each fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof; or for example, the optical detector can be configured to independently detect a plurality of fluorescent dyes at a plurality of different locations on a microfluidic cartridge, wherein each fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof in a different sample.

The heat source can be, for example, a heat source such as a resistive heater or network of resistive heaters, a

reversible heat source such as a liquid-filled heat transfer circuit or a thermoelectric element, a radiative heat source such as a xenon lamp, and the like.

In preferred embodiments, the at least one heat source can be a contact heat source selected from a resistive heater (or network thereof), a radiator, a fluidic heat exchanger and a Peltier device. The contact heat source can be configured at the receiving bay to be thermally coupled to one or more distinct locations of a microfluidic cartridge received in the bay, whereby the distinct locations are selectively heated. At least one additional contact heat source can be included, wherein the contact heat sources are each configured at the bay to be independently thermally coupled to a different distinct location in a microfluidic cartridge received in the bay, whereby the distinct locations are independently heated. The contact heat source can be configured to be in direct physical contact with a distinct location of a microfluidic cartridge received in the bay. In various embodiments, each contact source heater can be configured to heat a distinct location having an average diameter in 2 dimensions from about 1 millimeter (mm) to about 15 mm (typically about 1 mm to about 10 mm), or a distinct location having a surface area of between about 1 mm² about 225 mm² (typically between about 1 mm² and about 100 mm², or in some embodiments between about 5 mm² and about 50 mm²).

In various embodiments, at least one heat source can be a radiative heat source configured to direct heat to a distinct location of a microfluidic cartridge received in the receiving bay.

In various embodiments, the apparatus includes one or more force members that are configured to apply force to thermally couple the at least one heat source to at least a portion of the microfluidic cartridge received in the bay. The one or more force members can be configured to operate a mechanical member at the microfluidic cartridge. At least one force member can be manually operated. At least one force member can be mechanically coupled to a lid at the receiving bay, whereby operation of the lid operates the force member.

In various embodiments, the force applied by the one or more force members can result in an average pressure at an interface between a portion of the receiving bay and a portion of the microfluidic cartridge of about 1 psi. The application of force is important to ensure consistent thermal contact between the heater wafer and the PCR reactor and microvalves in the microfluidic cartridge.

In various embodiments, the apparatus can further include a lid at the receiving bay, the lid being operable to at least partially exclude ambient light from the bay. The lid can be, for example, a sliding lid. The lid can include the optical detector. A major face of the lid at the bay can vary from planarity by less than about 100 micrometers, for example, less than about 25 micrometers. The lid can be configured to be removable from the apparatus. The lid can include latching member that ensures that the lid is securely closed before amplification reactions are applied to the samples in the cartridge.

FIG. 35 shows a schematic cross-sectional view of a part of an apparatus as described herein, showing input of sample into a cartridge **200** via a pipette lip **10** (such as a disposable pipette) attached to an automated dispensing head, and an inlet **202**. Although not shown, there are as many inlets **202** as samples to be input into cartridge **200**. Inlet **202** is preferably configured to receive a pipette or the bottom end of a PCR tube and thereby accept sample for analysis with minimum waste, and with minimum introduction of air. Cartridge **200** is disposed on top of and in contact with a

heater substrate **400**. Read head **300** is positioned above cartridge **200** and a cover for optics **310** restricts the amount of ambient light that can be detected by the read head.

In various embodiments, a system as described herein can include both a microfluidic cartridge and the diagnostic apparatus.

Microfluidic Cartridge

One aspect of the present technology relates to a microfluidic cartridge including a first, second, and third, layers that together define a plurality of microfluidic networks, each network having various components configured to carry out PCR on a sample having one or more polynucleotides whose presence is to be determined. The cartridge includes one or more sample lanes in parallel, wherein each lane is independently associated with a given sample for simultaneous processing, and each lane contains an independently configured microfluidic network. An exemplary cartridge having such a construction is shown in FIG. **30**. Such a cartridge is simple to manufacture, and permits PCR in a concentrated reaction volume (~4 µl) and enables rapid thermocycling, as ~20 seconds per cycle.

Although other layers may be found in cartridges having comparable performance and ease of manufacture, the cartridge herein includes embodiments having only three layers in their construction: a substrate having an upper side and an opposed lower side, wherein the substrate comprises a microfluidic network having a plurality of sample lanes; a laminate attached to the lower side to seal the components of the microfluidic network, and provide an effective thermal transfer layer between a dedicated heating element and components in the microfluidic network; and a label, attached to the upper side that also covers and seals holes that are used in the manufacturing process to load microfluidic components such as valves. Thus, embodiments herein include microfluidic cartridges consisting of three layers, a substrate, a laminate, and a label, though other, additional, features other than layers may be consistent with such characterizations. Embodiments herein further include microfluidic cartridges consisting essentially of three layers, a substrate, a laminate, and a label, though other, additional, features other than layers may be consistent with such characterizations. Furthermore, embodiments herein still further include microfluidic cartridges comprising three layers, a substrate, a laminate, and a label.

A microfluidic network can include, in fluidic communication, one or more components selected from the group consisting of: gates, valves such as thermally actuated valves, channels, vents, and reaction chambers. Particular components of exemplary microfluidic networks are further described elsewhere herein. The cartridge typically processes the sample by increasing the concentration of a polynucleotide to be determined.

A sample lane is a set of elements, controllable independently of those in another sample lane, by which a sample can be accepted and analyzed, according to methods described herein. A lane comprises at least a sample inlet, and a microfluidic component, as further described herein in connection with a microfluidic cartridge. In some embodiments, each microfluidic network additionally comprises an overflow reservoir to contain extra liquid dispensed into the cartridge.

In various embodiments, a lane can include a sample inlet port, a first thermally actuated valve, a second thermally actuated valve, a PCR reaction chamber, and channels connecting the inlet port to the PCR reaction chamber via the first valve, and channels connecting the PCR reaction chamber to an exit vent via the second valve. The sample inlet

valve can be configured to accept a quantity of sample at a pressure differential compared to ambient pressure of between about 100 to 5000 Pa. It should be noted that the lower the loading pressure, the higher the fill time for a aliquot of reaction mix to fill the microfluidic network. Applying more pressure will reduce the fill time, but if the time for which the pressure is applied is not determined correctly, the sample could be blown out through the microfluidic cartridge (if an end hydrophobic vent is not present). Therefore the time for which the pressure is applied should to be properly determined, such as by methods available to one of ordinary skill in the art, to prevent underfill or overflow. In general, the fill time is inversely proportional to the viscosity of the solution. For example, FIG. **37** shows a microfluidic cartridge containing twelve independent sample lanes capable of independent (simultaneous or successive) processing of samples.

The microfluidic network in each lane is typically configured to carry out PCR on a PCR-ready sample, such as one containing nucleic acid (DNA or RNA) extracted from a raw biological sample using other aspects of the apparatus as further described herein. A PCR-ready sample is thus typically a mixture comprising the PCR reagent(s) and the neutralized polynucleotide sample, suitable for subjecting to thermal cycling conditions that create PCR amplicons from the neutralized polynucleotide sample. For example, a PCR-ready sample can include a PCR reagent mixture comprising a polymerase enzyme, a positive control plasmid, a fluorogenic hybridization probe selective for at least a portion of the plasmid and a plurality of nucleotides, and at least one probe that is selective for a polynucleotide sequence.

Typically, the microfluidic network is configured so that the time required for a microdroplet of sample to pass from the inlet to the second valve is less than 50% of the time required for the sample to travel up to the exit vent. Typically, the microfluidic network is designed to have an increased flow resistance downstream of the two valves without increasing the total volume of the microfluidic network in comparison to the amount required to fill from the first valve to the end vent of the network.

FIG. **38A** shows a perspective view of a portion of an exemplary microfluidic cartridge **200** according to the present technology. The cartridge may be referred to as a multi lane PCR cartridge with dedicated pipette inlets **202**. Shown in FIG. **38A** are various representative components of cartridge **200**. For example, sample inlet **202** is configured to accept a syringe, a pipette, or a PCR tube containing a PCR ready sample. More than one inlet **202** is shown, wherein one inlet operates in conjunction with a single lane. Various components of microfluidic circuitry in each lane are also visible. For example, microvalves **204**, and **206**, and vents **208**. ore parts of microfluidic circuitry in a given lane. Also shown is an ultrafast PCR reactor **210**, which, as further described herein, is a microfluidic channel that is long enough to permit PCR to occur in a sample. Above PCR reactor **210** is a window **212** that permits optical detection, such as detection of fluorescence from a fluorescent substance, such as a fluorogenic hybridization probe, in PCR reactor **210** when a detector is situated above window **212**.

A multi-lane cartridge is configured to accept a number of samples, in particular embodiments 12 samples, wherein the samples include at least a first sample and a second sample, wherein the first sample and the second sample each contain one or more polynucleotides in a form suitable for amplification. The polynucleotides in question may be the same as, or different from one another, in different lanes of a cartridge. The multi-sample cartridge comprises at least a

US 10,632,466 B1

37

first microfluidic network and a second microfluidic network, adjacent to one another, wherein each of the first microfluidic network and the second microfluidic network is as elsewhere described herein, and wherein the first microfluidic network accepts the first sample, and wherein the second microfluidic network accepts the second sample.

The sample inlets of adjacent lanes are reasonably spaced apart from one another to prevent any contamination of one sample inlet from another sample when a user introduces a sample into any one cartridge. In some embodiments, the sample inlets are configured so as to prevent subsequent inadvertent introduction of sample into a given lane after a sample has already been introduced into that lane.

In some embodiments, the multi-sample cartridge has a size substantially the same as that of a 96-well plate as is customarily used in the art. Advantageously, then, the cartridge may be used with plate handlers used elsewhere in the art. Still more preferably, however, the multi-sample cartridge is designed so that a spacing between the centroids of sample inlets is 9 mm, which is an industry-recognized standard. This means that, in certain embodiments the center-to-center distance between inlet holes in the cartridge that accept samples from PCR tubes, as further described herein, is 9 mm. The inlet holes are manufactured frustoconical in shape with an appropriate conical angle so that industry-standard pipette tips (2 μ l, 20 μ l, 200 μ l, volumes, etc.) fit snugly, entering from the widest point of the inlet. Thus, in certain embodiments, an inlet comprises an inverted frustoconical structure of at least 1 mm height, and having a diameter at its widest point that accepts entry of a pipette tip, of from 1-5 mm. The apparatus herein may be adapted to suit other, later-arising, industry standards for pipette tips not otherwise described herein. Typically the volume of sample accepted via an inlet into a microfluidic network in a sample lane is from 1-20 μ l, and may be from 3-5 μ l. The inlet hole can be designed to fit a pipette tip snugly and to create a good seal around the pipette tip, within the cone of the inlet hole. However, the cone is designed such that the sealing is reversible because it is undesirable if the seal is so tight that the cartridge can be pulled away from its tray, or location in the receiving bay, when the pipette tips are lifted after the dispensing operations.

FIG. 37 shows a plan view of an exemplary microfluidic cartridge having 12 lanes. The inlet ports have a 6 mm spacing, so that, when used in conjunction with an automated sample loader having 4 heads, spaced equidistantly at 9 mm apart, the inlets can be loaded in three batches of 4 inlets, e.g., inlets 1, 4, 7, and 10 together, followed by 2, 5, 8, and 11, then finally 3, 6, 9, and 12, wherein the 12 inlets are numbered consecutively from one side of the cartridge to the other.

FIG. 39A shows a plan view of a representative microfluidic circuit found in one lane of a multi-lane cartridge such as shown in FIGS. 38A and 38B. FIG. 39B shows another plan view (left panel) of another representative microfluidic circuit found in one lane of a multi-lane cartridge such as shown in FIG. 36, and shows how the circuit is visible through the cartridge construction (right panel). Other configurations of microfluidic network would be consistent with the function of the cartridges and apparatus described herein. In sequence, sample is introduced through liquid inlet 202, and optionally flows into a bubble removal vent channel 208 (which permits adventitious air bubbles introduced into the sample during entry, to escape), and continues along a channel 216. Typically, when using a robotic dispenser of liquid sample, the volume is dispensed

38

accurately enough that formation of bubbles is not a significant problem, and the presence of vent channel 208 is not necessary.

Throughout the operation of cartridge 200 the fluid is manipulated as a microdroplet (not shown in FIGS. 39A, B). Valves 204 and 206 are shown in FIG. 39A as double-valves, having a source of thermally responsive material (also referred to as a temperature responsive substance) on either side of the channel where they are situated. However, valves 204 and 206 may either or both be single valves that have a source of thermally responsive material on only one side of the respective channels. Valves 204 and 206 are initially open, so that a microdroplet of sample-containing fluid can be pumped into PCR reactor 210 from inlet hole 202. Upon initiating of processing, the detector present on top of the PCR reactor checks for the presence of liquid in the PCR reactor, and then closes valves 204 and 206 to isolate the PCR reaction mix from the channels on either side.

The PCR reactor 210 is a microfluidic channel that is heated through a series of cycles to carry out amplification of nucleotides in the sample, as further described herein. Typically the PCR reactor has a volume of 3-5 μ l, in particular, 4 μ l. The inside walls of the channel in the PCR reactor are made very smooth and polished to a shiny finish (for example, using a polish selected from SPI A1, SPI A2, SPI A3, SPI B1, or SPI B2) during manufacture. This is in order to minimize any microscopic air trapping in the surface of the PCR reactor, which would cause bubbling during the thermocycling steps. The presence of bubbles especially in the detection region of the PCR reactor might cause a false reading for the PCR reaction. Furthermore, the PCR reactor 210 is made shallow such that the temperature gradient across the depth of the channel is minimized. The region of the cartridge 212 above PCR reactor 210 permits a detector to monitor progress of the reaction and also to detect fluorescence from a probe that binds to a quantity of amplified nucleotide. The region 212 is made of thinner material than the rest of the cartridge so as to permit the PCR reactor to be more responsive to a heating cycle (for example, to rapidly heat and cool between temperatures appropriate for denaturing and annealing steps), and so as to reduce glare, autofluorescence, and undue absorption of fluorescence. Both valves 204 and 206 are closed prior to thermocycling to prevent any evaporation of liquid, bubble generation, or movement of fluid from the PCR reactor.

End vent 214 prevents a user from introducing any excess amount of liquid into the microfluidic cartridge, as well as playing a role of containing any sample from spilling over to unintended parts of the cartridge. A user may input sample volumes as small as an amount to fill from the bubble removal vent to the middle of the PCR reactor, or up to valve 204 or beyond valve 204. The use of micro valves prevents both loss of liquid or vapor thereby enabling even a partially filled reactor to successfully complete a PCR thermocycling reaction. The application of pressure (such as ~1 psi) to contact the cartridge to the heater of the instrument assists in achieving better thermal contact between the heater and the heat-receivable parts of the cartridge, and also prevents the bottom laminate structure from expanding, as would happen if the PCR channel was partially filled with liquid and the entrapped air would be thermally expanded during thermocycling.

In various embodiments, the microfluidic network can optionally include at least one hydrophobic vent additional to the end vent.

After PCR has been carried out on a sample, and presence or absence of a polynucleotide of interest has been deter-

mined, it is preferred that the amplified sample remains on the cartridge and that the cartridge is either used again (if one or more lanes remain open), or disposed of. Should a user wish to run a post amplification analysis, such as gel electrophoresis, the user may pierce a hole through the laminate of the cartridge, and recover an amount—typically about 1.5 microliter—of PCR product. The user may also place the individual PCR lane on a special narrow heated plate, maintained at a temperature to melt the wax in the valve, and then aspirate the reacted sample from the inlet hole of that PCR lane.

In various embodiments, the microfluidic network can optionally include at least one reservoir configured to contain waste.

In various embodiments, the microfluidic cartridge can further include a label, such as a computer-readable or scannable label. For example, the label can be a bar code, a radio frequency tag, or one or more computer-readable, or optically scannable, characters. The label can be positioned such that can be read by a sample identification verifier as further described herein.

In various embodiments, during transport and storage, the microfluidic cartridge can be further surrounded by a sealed pouch. The microfluidic cartridge can be sealed in the pouch with an inert gas. The microfluidic cartridge can be disposable.

Microfluidic cartridge 200 can be fabricated as desired. Typically, the microfluidic cartridge layer includes a layer of polypropylene or other plastic label with pressure sensitive adhesive (typically between about 50 and 150 microns thick) configured to seal the wax loading holes of the valves, trap air used for valve actuation, and serve as a location for operator markings. This layer can be in two separate pieces, though it would be understood by one of ordinary skill in the art that in many embodiments a single piece layer would be appropriate.

The microfluidic substrate layer, is typically injection molded out of a plastic, preferably a zeonor plastic (cyclic olefin polymer), having a PCR channel and valve channels on a first side, and vent channels and various inlet holes, including wax loading holes and liquid inlet holes, on a second side (disposed toward the label). Typically, all of the microfluidic networks together, including the PCR reactors, the inlet holes and the valves for isolating the PCR reaction chambers, are defined in a single substrate. The substrate is made of a material that confers rigidity on the substrate and cartridge, and is impervious to air or liquid, so that entry or exit of air or liquid during operation of the cartridge is only possible through the inlet or the vent.

Channels of a microfluidic network in a lane of cartridge 200 typically have at least one sub-millimeter cross-sectional dimension. For example, channels of such a network may have a width and/or a depth of about 1 mm or less (e.g., about 750 microns or less, about 500 microns, or less, about 250 microns or less).

The cartridge can further include a heat scalable laminate layer 222 (typically between about 100 and about 125 microns thick) attached to the bottom surface of the microfluidic substrate using, for example, heat bonding, pressure bonding, or a combination thereof. The laminate layer 222 may also be made from a material that has an adhesive coating on one side only, that side being the side that contacts the underside of the microfluidic substrate. This layer may be made from a single coated tape having a layer of Adhesive 420, made by 3M, Exemplary tapes include single-sided variants of double sided tapes having product

nos. 9783, 9795, and 9795B, and available from 3M. Other acceptable layers may include tapes based on micro-capsule based adhesives.

In use, cartridge 200 is typically thermally associated with an array of heat sources configured to operate the components (e.g., valves, gates, and processing region 210) of the device. In some embodiments, the heat sources are operated by an operating system, which operates the device during use. The operating system includes a processor (e.g., a computer) configured to actuate the heat sources according to a desired protocol. Processors configured to operate microfluidic devices are described in, e.g., U.S. application Ser. No. 09/819,105, filed Mar. 28, 2001, which application is incorporated herein by reference.

Table 1 outlines volumes, pumping pressures, and operation times associated with various components of a microfluidic cartridge.

TABLE 1

Operation	Pumping Pressure	Displacement Volume	Time of Operation
Mixing displacements	~2 psi	10-25 µl	1-2 minutes
Moving valve wax plugs	~1-2 psi	<1 µl	5-15 seconds

Operation	Pump Used	Pump Design	Pump Actuation
Mixing displacements	Expancel Pump	Same as above	Same as above
Moving valve wax plugs	Thermo-pneumatic pump	1 µl of trapped air	Heat trapped air to ~70-90 C.

In some embodiments, a microfluidic cartridge further comprises a registration member that ensures that the cartridge is received by a complementary diagnostic apparatus in a single orientation, for example, in a receiving bay of the apparatus. The registration member may be a simple cut-out from an edge or a corner of the cartridge (as shown in FIG. 38A), or may be a series of notches, or some other configuration of shapes that require a unique orientation of placement in the apparatus.

In some embodiments, the microfluidic cartridge comprises two or more positioning elements, or fiducials, for use when filling the valves with thermally responsive material. The positioning elements may be located on the substrate, typically the upper face thereof.

The microfluidic cartridges may also be stackable, such as for easy storage or transport, or may be configured to be received by a loading device, as further described herein, that holds a plurality of cartridges in close proximity to one another, but without being in contact. In order to accomplish either or both of these characteristics, the substrate may comprise two ridges, one of each situated along each of two opposite edges of the cartridge, the ridges disposed on the upper side of the substrate. Thus, where a cartridge has a rectangular aspect (ignoring any registration member or mechanical key), the two ridges may be situated along the long side, or along the short side, of the cartridge.

Valves

A valve is a microfluidic component that has a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). An exemplary double valve is shown in FIG. 40A. A double valve has two channels, one on either side of

the channel whose flow it regulates, whereas a single valve has just one channel, disposed on one side of the channel whose flow it regulates.

Upon actuation, e.g., by application of heat, the valve transitions to a closed state that prevents material, such as a microdroplet of PCR-ready sample, from passing along the channel from one side of the valve to the other. For example, a valve includes one or more masses of a thermally responsive substance (TRS) that is relatively immobile at a first temperature and more mobile at a second temperature. A mass of TRS can be an essentially solid mass or an agglomeration of smaller particles that cooperate to obstruct the passage upon actuation. Examples of TRS's include a eutectic alloy (e.g., a solder), wax (e.g., an olefin), polymers, plastics, and combinations thereof. The first and second temperatures are insufficiently high to damage materials, such as polymer layers of a microfluidic cartridge in which the valve is situated. Generally, the second temperature is less than about 90° C. and the first temperature is less than the second temperature (e.g., about 70° C. or less).

For each mass associated with a valve, a chamber is in gaseous communication with the mass. Upon heating gas (e.g., air) in the chamber(s) and heating the one or more masses of TRS to the second temperature, gas pressure within a chamber moves the corresponding mass into the channel obstructing material from passing therealong. Other valves of the network have the same structure and operate in the same fashion as the valves described herein.

In order to make the valve sealing very robust and reliable, the flow channel at the valve junction is made narrow (150 μ m wide and 150 μ m deep or narrower) and the constricted channel is made at least 0.5 or 1 mm long such that the wax seals up a long narrow channel thereby reducing any leakage through the walls of the channel. In the case of a bad seal, there is leakage of fluid around the walls of the channel, past the wax. So the flow channel is narrowed as much as possible, and made longer, e.g., as long as ~1 mm. The valve operates by heating air in the wax-loading port, which forces the wax forwards in a manner so that it does not come back to its original position. In this way, both air and wax are heated during operation of the valve.

In various embodiments, the microfluidic network can include a bent valve as shown in FIG. 32B (as a single valve) to reduce the footprint of the valve on the cartridge and hence reduce cost per part for manufacturing highly dense microfluidic substrates. In the valve of FIG. 40B, the loading hole for TRS is in the center of the valve; the structures at either end are an inlet and an outlet and are shown for illustrative purposes only. Single valve shown.

In various embodiments, the network can include a curved valve as shown in FIG. 40C. also as a single valve, in order to reduce the effective cross-section, of the micro-valve, enabling manufacture of cheaper dense microfluidic devices.

Vents

A hydrophobic vent (e.g., a vent in FIG. 41) is a structure that permits gas to exit a channel while limiting (e.g., preventing) liquid from exiting the channel. Typically, hydrophobic vents include a layer of porous hydrophobic material (e.g., a porous filter such as a porous hydrophobic membrane from Osmonics) that defines a wall of the channel. As discussed herein, hydrophobic vents can be used to position a microdroplet of sample at a desired location within a microfluidic network.

The hydrophobic vents of the cartridge are preferably constructed so that the amount of air that escapes through them is maximized while minimizing the volume of the

channel below the vent surface. Accordingly, it is preferable that the vent is constructed so as to have a hydrophobic membrane of huge surface area and a shallow cross section of the microchannel below the vent surface.

Bubble removal hydrophobic vents typically have a length of at least about 2.5 mm (e.g., at least about 5 mm, at least about 7.5 mm) along a channel. The length of the hydrophobic vent is typically at least about 5 times (e.g., at least about 10 times, at least about 20 times) larger than a depth of the channel within the hydrophobic vent. For example, in some embodiments, the channel depth within the hydrophobic vent is about 300 microns or less (e.g., about 250 microns or less, about 200 microns or less, about 150 microns or less). Bubble vents are optional in the microfluidic networks of the microfluidic cartridges described herein.

The depth of the channel within the hydrophobic vent is typically about 75% or less (e.g., about 65% or less, about 60% or less) of than the depth of the channel upstream and downstream of the hydrophobic vent. For example, in some embodiments the channel depth within the hydrophobic vent is about 150 microns and the channel depth upstream and downstream of the hydrophobic vent is about 250 microns.

A width of the channel within the hydrophobic vent is typically at least about 25% wider (e.g., at least about 50% wider) than a width of the channel upstream from the vent and downstream from the vent. For example, in an exemplary embodiment, the width of the channel within the hydrophobic vent is about 400 microns and the width of the channel upstream and downstream from the vent is about 250 microns.

Highly Multiplexed Embodiment

Embodiments of the apparatus and cartridge described herein may be constructed that have high-density microfluidic circuitry on a single cartridge that thereby permit processing of multiple samples in parallel, or in sequence, on a single cartridge. Preferred numbers of such multiple samples include 36, 40, 48, 50, 64, 72, 80, 96, and 100, but it would be understood that still other numbers are consistent with the apparatus and cartridge herein, where deemed convenient and practical.

Accordingly, different configurations of lanes, sample inlets, and associated heater networks are contemplated that can facilitate processing such numbers of samples on a single cartridge are within the scope of the instant disclosure. Similarly, alternative configurations of detectors for use in conjunction with such a highly multiplexed cartridge are also within the scope of the description herein.

In an exemplary embodiment, a highly multiplexed cartridge has 48 PCR channels, and has independent control of each valve in the channel, with 2 banks of thermocycling protocol per channel, as shown in FIG. 43. In the embodiment in FIG. 43, the heaters are arranged in three arrays. Heaters in two separate glass regions only apply heat to valves in the microfluidic networks in each lane. Because of the low thermal conductivity of glass, the individual valves may be heated separately from one another. This permits samples to be loaded into the cartridge at different times, and passed to the PCR reaction chambers independently of one another. The PCR heaters are mounted on a silicon substrate—and are not readily heated individually, but thereby permit batch processing of PCR samples, where multiple samples from different lanes are amplified by the same set of heating/cooling cycles. It is preferable for the PCR heaters to be arranged in 2 banks (the heater arrays on the left and right are not in electrical communication with one another), thereby permitting a separate degree of sample control.

FIG. 42 shows a representative cartridge, revealing an inlet configuration for a 48-sample cartridge. The inlet configuration is compatible with an automatic pipetting machine that has dispensing heads situated at a 9 mm spacing. For example, such a machine having 4 heads can load 4 inlets at once, in 12 discrete steps, for the cartridge of FIG. 42.

FIG. 44 shows, in close, up an exemplary spacing of valves and lanes in adjacent lanes of a multi-sample microfluidic cartridge.

FIGS. 45 and 46 show close-ups of, respectively, heater arrays, and inlets, of the exemplary cartridge shown in FIG. 44.

FIGS. 47A-47C show various views of an embodiment of a radially-configured highly-multiplexed cartridge, having a number of inlets, microfluidic lanes, and PCR reaction zones.

The various embodiments shown in FIGS. 42-47C are compatible with liquid dispensers, receiving bays, and detectors that are configured differently from the specific examples described herein.

In another preferred embodiment (not shown in the FIGS), a cartridge and apparatus is configured so that the read-head does not cover the sample inlets, thereby permitting loading of separate samples while other samples are undergoing PCR thermocycling.

Heater Configurations to Ensure Uniform Heating of a Region

Another aspect of the apparatus described hereto relates to a method and apparatus for uniformly controlling the heating of a region of a microfluidic network that includes but is not limited to one or more microfluidic components. In an exemplary embodiment, multiple heaters can be configured to simultaneously and uniformly heat a region, such as the PCR reaction zone, of the microfluidic cartridge.

In preferred embodiments, a microfluidic cartridge having a microfluidic network comprising one or more microfluidic components is brought into contact with a heat source, within a suitably configured apparatus. The heat source is configured so that particular heating elements are situated to heat specific components of the microfluidic network of the cartridge.

FIG. 48 shows a cross-sectional view of an exemplary microfluidic cartridge to show relative location of PCR channel in relation to the heaters when the cartridge is placed in the instrument. The view in FIG. 48 is also referred to as a sectional-isometric view of the cartridge lying over the heater wafer. A window 903 above the PCR channel in the cartridge is shown in perspective view. PCR channel 901 (for example, 150 μ deep x 700 μ wide), is shown in an upper layer of the cartridge. A laminate layer 905 of the cartridge (for example, 125 μ thick) is directly under the PCR channel 901. A further layer of thermal interlace laminate 907 on the cartridge (for example, 125 μ thick) lies directly under the laminate layer 905. Heaters are situated in a further layer 913 directly under the thermal interface laminate. The heaters are photolithographically defined and etched metal layers of gold (typically about 3,000 Å thick). Layers of 400 \AA of TiW are deposited on top and bottom of the gold layer to serve as an adhesion layer. The substrate used is glass, fused silica or quartz wafer having a thickness of 0.4 mm, 0.5 mm or 0.7 mm or 1 mm. A thin electrically-insulative layer of 2 μ m silicon oxide serves as an insulative layer on top of the metal layer. Additional thin electrically insulative layers such as 2-4 μ m of Parylene may also be deposited on top of the Silicon oxide surface. Two long heaters 909 and 911, as further described herein, are also shown.

Referring to FIGS. 49A and 49B, the PCR reaction zone 1001, typically having a volume ~ 1.6 μ l, is configured with a long side and a short side, each with an associated heating element. The apparatus therefore preferably includes four heaters disposed along the sides of, and configured to heat, the PCR reaction zone, as shown in the exemplary embodiment of FIG. 38A; long top heater 1005, long bottom heater 1003, short left heater 1007, and short right heater 1009. The small gap between long top heater 1005 and long bottom heater 1003 results in a negligible temperature gradient (less than 1° C. across the width of the PCR channel at any point along the length of the PCR reaction zone) and therefore an effectively uniform temperature throughout the PCR reaction zone. The heaters on the short edges of the PCR reactor provide heat to counteract the gradient created by the two long heaters from the center of the reactor to the edge of the reactor. It would be understood by one of ordinary skill in the art that still other configurations of one or more heater(s) situated about a PCR reaction zone are consistent with the methods and apparatus described herein. For example, a 'long' side of the reaction zone can be configured to be heated by two or more heaters. Specific orientations and configurations of heaters are used to create uniform zones of heating even on substrates having poor thermal conductivity because the poor thermal conductivity of glass, or quartz, or fused silica substrates is utilized to help in the independent operation of various microfluidic components such as valves and independent operation of the various PCR lanes.

In preferred embodiments, each heater has an associated temperature sensor. In the embodiment of FIG. 49A, a single temperature sensor 1011 is used for both long heaters. A temperature sensor 1013 for short left heater, and a temperature sensor 1015 for short right heater are also shown. The temperature sensor in the middle of the reactor is used to provide feedback and control the amount of power supplied to the two long heaters, wherein each of the short heaters has a dedicated temperature sensor placed adjacent to it in order to control it. As further described herein, temperature sensors are preferably configured to transmit information about temperature in their vicinity to the processor at such times as the heaters are not receiving current that causes them to heat. This can be achieved with appropriate control of current cycles.

In order to reduce the number of sensor or heater elements required to control a PCR heater, we may use the heaters to sense as well as heat, and thereby obviate the need to have a separate dedicated sensor for each heater. In another embodiment, each of the four heaters may be designed to have an appropriate wattage, and connect the four heaters in series or in parallel to reduce the number of electronically-controllable elements from 4 to just 1, thereby reducing the burden on the electronics.

FIG. 49B shows expanded views of heaters and temperature sensors used in conjunction with a PCR reaction zone of FIG. 49A. Temperature sensors 1001 and 1013 are designed to have a room temperature resistance of approximately 200-300 ohms. This value of resistance is determined by controlling the thickness of the metal layer deposited (e.g., a sandwich of 400 Å TiW/3000 Å Au/400 Å TiW), and etching the winding metal line to have a width of approximately 10-25 μ m and 20-40 mm length. The use of metal in this layer gives it a temperature coefficient of resistivity of the order of 0.5-20° C./ohms, preferably in the range of 15-3° C./ohms. Measuring the resistance at higher temperatures will enable determination of the exact temperature of the location of these sensors.

US 10,632,466 B1

45

The configuration for uniform heating, shown in FIG. 49A for a single PCR reaction zone, can be applied to a multi-lane PCR cartridge in which multiple independent PCR reactions occur.

Each heater can be independently controlled by a processor and/or control circuitry used in conjunction with the apparatus described herein. FIG. 50 shows thermal images, from the top surface of a microfluidic cartridge having heaters configured as in FIGS. 49A and 49B, when each heater in turn is activated, as follows: (A): Long Top only; (B) Long Bottom only; (C) Short Left only; (D) Short Right only; and (E) All Four Heaters on. Panel (F) shows a view of the reaction zone and heaters on the same scale as the other image panels in FIG. 50. Also shown in the figure is a temperature bar.

Use of Cutaways in Cartridge Substrate to Improve Rate of Cooling During PCR Cycling

During a PCR amplification of a nucleotide sample, a number of thermal cycles are carried out. For improved efficiency, the cooling between each application of heat is preferably as rapid as possible. Improved rate of cooling can be achieved with various modifications to the heating substrate, as shown in FIGS. 51A-51C.

One way to achieve rapid cooling is to cut away portions of the microfluidic cartridge substrate, as shown in FIG. 51A. The upper panel of FIG. 51A is a cross-section of an exemplary microfluidic cartridge taken along the dashed line A-A' as marked on the lower panel of FIG. 51A. PCR reaction zone 901, and representative heaters 1003 are shown. Also shown are two cutaway portions, one of which labeled 1201, that are situated alongside the heaters that are situated along the long side of the PCR reaction zone. Cutaway portions such as 1201 reduce the thermal mass of the cartridge, and also permit air to circulate within the cutaway portions. Both of these aspects permit heat to be conducted away quickly from the immediate vicinity of the PCR reaction zone. Other configurations of cutouts, such as in shape, position, and number, are consistent with the present technology.

Another way to achieve rapid cooling is to cutaway portions of the heater substrate, as shown in FIG. 51B. The lower panel of FIG. 51B is a cross-section of an exemplary microfluidic cartridge and heater substrate taken along the dashed line A-A' as marked on the upper panel of FIG. 51B. PCR reaction zone 901, and representative heaters 1003 are shown. Also shown are four cutaway portions, one of which labeled 1205, that are situated alongside the heaters that are situated along the long side of the PCR reaction zone. Cutaway portions such as 1205 reduce the thermal mass of the heater substrate, and also permit air to circulate within the cutaway portions. Both of these aspects permit heat to be conducted away quickly from the immediate vicinity of the PCR reaction zone. Four separate cutaway portions are shown in FIG. 51B so that control circuitry to the various heaters is not disrupted. Other configurations of cutouts, such as in shape, position, and number, are consistent with the present technology. These cutouts may be created by a method selected from: selective etching using wet etching processes, deep reactive ion etching, selective etching using CO₂ laser or femtosecond laser (to prevent surface cracks or stress near the surface), selective mechanical drilling, selective ultrasonic drilling, or selective abrasive particle blasting. Care has to be taken to maintain mechanically integrity of the heater while reducing as much material as possible.

FIG. A1 shows a combination of cutouts and use of ambient air cooling to increase the cooling rate during the cooling stage of thermocycling. A substantial amount of

46

cooling happens by convective loss from the bottom surface of the heater surface to ambient air. The driving force for this convective loss is the differential in temperatures between the glass surface and the air temperature. By decreasing the ambient air temperature by use of, for example, a peltier cooler, the rate of cooling can be increased. The convective heat loss may also be increased by keeping the air at a velocity higher than zero

An example of thermal cycling performance obtained with a configuration as described herein, is shown in FIG. 52 for a protocol that is set to heat up to 92° C., and stay there for 1 second, then cool to 62° C. and stay for 10 seconds. Cycle time is about 29 seconds, with 8 seconds required to heat from 62° C. and stabilize at 92° C., and 10 seconds required to cool from 92° C. and stabilize at 62° C.

Manufacturing Process for Cartridge

FIG. 53 shows a flow-chart 2800 for an assembly process for an exemplary cartridge as further described herein. It would be understood by one of ordinary skill in the art, both that various steps may be performed in a different order from that set forth in FIG. 53, and additionally that any given step may be carried out by alternative methods to those set forth in the figure. It would also be understood that, where separate steps are illustrated for carrying out two or more functions, such functions may be performed synchronously and combined into single steps and be consistent with the overall process described herein.

At 2802, a laminate layer is applied to a microfluidic substrate that has previously been engineered to have a microfluidic network constructed in it; edges are trimmed from the laminate where they spill over the bounds of the substrate.

At 2804, wax is dispensed and loaded into the micro valves of the microfluidic network in the microfluidic substrate. An exemplary process for carrying this out is further described herein.

At 2806, the cartridge is inspected to ensure that wax from step 2804 is loaded properly and that the laminate from step 2802 adheres properly to the microfluidic substrate. If a substrate does not satisfy either or both of these tests, it is discarded. If substrates repeatedly fail either or both of these tests, then the wax dispensing, or laminate application steps, as applicable, are reviewed.

At 2808, a hydrophobic vent membrane is applied to, and heat bonded to, the top of the microfluidic substrate over the wax valves, and on the opposite face of the substrate from the laminate. Edges of the membrane that are in excess of the boundary of the substrate are trimmed.

At 2810, the assembly is inspected to ensure that the hydrophobic vent membrane is bonded well to the microfluidic substrate without heat-clogging the microfluidic channels. If any of the channels is blocked, or if the bond between the membrane and the substrate is imperfect, the assembly is discarded, and, in the case of repeated discard events, the foregoing process step is reviewed.

At 2812, a thermally conductive pad layer is applied to the bottom laminate of the cartridge.

At 2814, two label strips are applied to the top of the microfluidic substrate, one to cover the valves, and a second to protect the vent membranes. It would be understood that a single label strip may be devised to fulfill both of these roles.

At 2816, additional labels are printed or applied to show identifying characteristics, such as a barcode #, lot# and expiry date on the cartridge. Preferably one or more of these labels has a space and a writable surface that permits a user to make an identifying annotation on the label, by hand.

US 10,632,466 B1

47

At **2818**, to facilitate transport and delivery to a customer, assembled and labeled cartridges are stacked and pack cartridges in groups, such as groups of 25, or groups of 10, or groups of 20, or groups of 50. Preferably the packaging is via an inert and/or moisture-free medium. ps Exemplary Wax-Deposition Process

Deposition of wax in valves of the microfluidic network, as at step **2804** may be carried out with the exemplary equipment shown in FIGS. **54A** and **54B**. The DispenseJet Series DJ-9000 (FIGS. **54A** and **54B**) is a non-contact dispenser that provides high-speed delivery and exceptional volumetric control for various fluids, including surface mount adhesive, underfill, encapsulants, conformal coating, UV adhesives, and silver epoxy. The DJ-9000 jets in tight spaces as small as 200 micrometers and creates fillet wet-out widths as small as 300 micrometers on the dispensed side of a substrate such as a die. It dispenses fluid either as discrete dots or a rapid succession of dots to form a 100-micron (4 mil) diameter stream of fluid from the nozzle. It is fully compatible with other commercially available systems such as the Asymtek Century C-718/C-720, Millennium M-2000, and Axiom X-1000 Series Dispensing Systems.

A DJ-9000 is manufactured by Asymtek under manufacturing quality control standards aim to provide precise and reliable performance. Representative specifications of the apparatus are as follows.

Characteristic	Specification
Size	Width: 35 mm Height: 110 mm Depth: 100 mm
Weight	400 grams - dry
Feed Tube Assembly	Nylon - Fitting Polyurethane - Tube
Fluid Chamber	Type 303 Stainless Steel
Seat and Nozzle	300/400 Series S/S, Carbide
Needle Assembly	52100 Bearing Steel - Shaft Hard Chrome Plate Carbide - Tip
Fluid Seal	PEEK/Stainless Steel
Fluid Chamber O-Ring	Ethylene Propylene
Jet Body	6061-T6 Aluminum Nickel Plated
Needle Assembly Bearings	PEEK
Thermal Control Body	6061-T6 Aluminum Nickel Plated
Reservoir Holder	Acetyl
Reservoir Size	5, 10, or 30 cc (0.17, 0.34, or 1.0 oz)
Feed Tube Assembly Fitting	Female Luer per ANSI/HIMA MD70.1-1983
Maximum Cycle Frequency	200 Hz
Minimum Valve Air Pressure	5.5 bar (80 psi)
Operating Noise Level	70 db*
Solenoid	24 VDC, 12.7 Watts
Thermal Control Heater	24 VDC, 14.7 Watts, 40 ohms
Thermal Control RTD	100 ohm, platinum
Maximum Heater Set Point	80 C.

*At Maximum Cycle Rate

An exploded view of this apparatus is shown in FIG. **54B**. Theory of Operation of DJ-9000

The DJ-9000 has a normally closed, air-actuated, spring-return mechanism, which uses momentum transfer principles to expel precise volumes of material. Pressurized air is regulated by a high-speed solenoid to retract a needle assembly from the seat. Fluid, fed into the fluid chamber, flows over the seat. When the air is exhausted, the needle

48

travels rapidly to the closed position, displacing fluid through the seat and nozzle in the form of a droplet. Multiple droplets fired in succession can be used to form larger dispense volumes and lines when combined with the motion of a dispenser robot.

The equipment has various adjustable features: The following features affect performance of the DJ-9000 and are typically adjusted to fit specific process conditions.

Fluid Pressure should be set so that fluid fills to the seat, but should not be influential in pushing the fluid through the seat and nozzle. In general, higher fluid pressure results in a larger volume of material jetted.

The Stroke Adjustment controls the travel distance of the Needle Assembly. The control is turned counterclockwise to increase needle assembly travel, or turned clockwise to decrease travel. An increase of travel distance will often result in a larger volume of material jetted.

The Solenoid Valve controls the valve operation. When energized, it allows air in the jet air chamber to compress a spring and thereby raise the Needle Assembly. When de-energized, the air is released and the spring forces the piston down so that the needle tip contacts the seat.

The seat and nozzle geometry are typically the main factors controlling dispensed material volume. The seat and nozzle size are determined based on the application and fluid properties. Other parameters are adjusted in accordance with seat and nozzle choices. Available seat and nozzle sizes are listed in the table hereinbelow.

Thermal Control Assembly: Fluid temperature often influences fluid viscosity and flow characteristics. The DJ-9000 is equipped with a Thermal Control Assembly that assures a constant fluid temperature.

Dot and Line Parameters: In addition to the DJ-9000 hardware configuration and settings, Dot and Line Parameters are set in a software program (referred to as FmNT) to control the size and quality of dots and lines dispensed.

Wax Loading in Valves

FIGS. **55A** and **55B** show how a combination of controlled hot drop dispensing into a heated microchannel device of the right dimensions and geometry is used to accurately load wax into a microchannel of a microfluidic cartridge to form a valve. The heated dispenser head can be accurately position over an inlet hole of the microchannel in the microfluidic device, and can dispense molten wax drops in volumes as small as 75 nanoliters with an accuracy of 20%. The inlet hole of the microchannel device is dimensioned in such a way that the droplet of 75 nl can be accurately shot to the bottom of the inlet hole using, for example, compressed air, or in a manner similar to an inkjet printing method. The microchannel device is maintained at a temperature above the melting point of the wax thereby permitting the wax to stay in a molten state immediately after it is dispensed. After the drop falls to the bottom of the inlet hole, the molten wax is drawn into the narrow channel by capillary action. The volume of the narrow section is designed to be approximately equal to a maximum typical amount that is dispensed into the inlet hole.

Heater Multiplexing (under software control)

Another aspect of the apparatus described herein, relates to a method for controlling the heat within the system and its components, as illustrated in FIG. **56**. The method leads to a greater energy efficiency of the apparatus described herein, because not all heaters are heating at the same time, and a given heater is receiving current for only part of the time.

Generally, the heating of microfluidic components, such as a PCR reaction zone, is controlled by passing currents through suitably configured microfabricated heaters. The heating can be further controlled by periodically turning the current on and off with varying pulse width modulation

US 10,632,466 B1

49

(PWM), wherein pulse width modulation refers to the on-time/off-time ratio for the current. The current can be supplied by connecting micro fabricated heater to a high voltage source (for example, 30V), which can be gated by the PWM signal. In some embodiments, the device includes 48 PWM signal generators. Operation of a PWM generator includes generating a signal with a chosen, programmable period (the end count) and granularity. For instance, the signal can be 4000 μ s (micro-seconds) with a granularity of 1 μ s, in which case the PWM generator can maintain a counter beginning at zero and advancing in increments of 1 μ s until it reaches 4000 μ s, when it returns to zero. Thus, the amount of heat produced can be adjusted by adjusting the end count. A high end count corresponds to a greater length of time during which the microfabricated heater receives current and therefore a greater amount of heat produced.

In various embodiments, the operation of a PWM generator can also include a programmable start count in addition to the aforementioned end count and granularity. In such embodiments, multiple PWM generators can produce signals that can be selectively non-overlapping (e.g., by multiplexing the on-time of the various heaters) such that the current capacity of the high voltage power is not exceeded. Multiple heaters can be controlled by different PWM signal generators with varying start and end counts. The heaters can be divided into banks, whereby a bank defines a group of heaters of the same start count. For example, 36 PWM generators can be grouped into six different banks, each corresponding to a certain portion of the PWM cycle (500 ms for this example). The end count for each PWM generator can be selectively programmed such that not more than six heaters will be on at any given time. A portion of a PWM cycle can be selected as dead time (count 3000 to 4000 for this example) during which no heating takes place and sensitive temperature sensing circuits can use this time to sense the temperature. The table below represents a PWM cycle for the foregoing example:

	Start Count	End Count	Max End count
Bank 1			
PWM generator #1	0	150	500
PWM generator #2	0	220	500
...
PWM generator #6	0	376	500
Bank 2			
PWM generator #7	500	704	1000
PWM generator #8	500	676	1000
...
PWM generator #12	500	780	1000
Bank 3			
PWM generator #13	1000	1240	1500
PWM generator #14	1000	1101	1500
...
PWM generator #18	1000	1409	1500
Bank 4			
PWM generator #19	1500	1679	2000
PWM generator #20	1500	1989	2000
...
PWM generator #24	1500	1502	2000
Bank 5			
PWM generator #25	2000	2090	2500
PWM generator #26	2000	2499	2500
...
PWM generator #30	2000	2301	2500

50

-continued

	Start Count	End Count	Max End count
Bank 6			
PWM generator #31	2500	2569	3000
PWM generator #32	2500	2790	3000
...
PWM generator #36	2500	2678	3000

Use of Detection System to Measure/Detect Fluid in PCR Chamber

The apparatus optionally has a very sensitive fluorescence detector that is able to collect fluorescence light from the PCR chamber 210 of a microfluidic cartridge. This detector is used to detect the presence of liquid in the chamber, a measurement that determines whether or not to carry out a PCR cycle. A background reading is taken prior to filling the chamber with liquid. Another reading is taken after microfluidic operations have been performed that should result in filling the PCR chamber with liquid. The presence of liquid alters the fluorescence reading from the chamber. A programmable threshold value is used to tune an algorithm programmed into the processor (for example, the second reading has to exceed the first reading by 20%). If the two readings do not differ beyond the programmed margin, the liquid is deemed to not have entered the chamber, and a PCR cycle is not initiated for that chamber, instead, a warning is issued to a user.

Computer Program Product

In various embodiments, a computer program product for use with the apparatus herein includes computer readable instructions thereon for operating the apparatus.

In various embodiments, the computer program product can include one or more instructions to cause the system to: output an indicator of the placement of the microfluidic cartridge in the bay; read a sample label or a microfluidic cartridge label; output directions for a user to input a sample identifier; output directions for a user to load a sample transfer member with the PCR ready sample; output directions for a user to introduce the PCR-ready sample into the microfluidic cartridge; output directions for a user to place the microfluidic cartridge in the receiving bay; output directions for a user to close the lid to operate the force member; output directions for a user to pressurize the PCR-ready sample in the microfluidic cartridge by injecting the PCR-ready sample with a volume of air between about 0.5 ml, and about 5 ml.; and output status information for sample progress from one or more lanes of the cartridge.

In various embodiments, the computer program product can include one or more instructions to cause the system to: heat the PCR ready-sample under thermal cycling conditions suitable for creating PCR amplicons from the neutralized polynucleotide; contact the neutralized polynucleotide sample or a PCR amplicon thereof with at least one probe that is selective for a polynucleotide sequence; independently contact each of the neutralized polynucleotide sample and a negative control polynucleotide with the PCR reagent mixture under thermal cycling conditions suitable for independently creating PCR amplicons of the neutralized polynucleotide sample and PCR amplicons of the negative control polynucleotide; contact the neutralized polynucleotide sample or a PCR amplicon thereof and the negative control polynucleotide or a PCR amplicon thereof with at least one probe that is selective for a polynucleotide sequence; output a determination of the presence of a

US 10,632,466 B1

51

polynucleotide sequence in the biological sample, the polynucleotide sequence corresponding to the probe, if the probe is detected in the neutralized polynucleotide sample or a PCR amplicon thereof; and/or output a determination of a contaminated result if the probe is detected in the negative control polynucleotide or a PCR amplicon thereof.

In various embodiments, the computer program product can include one or more instructions to cause the system to automatically conduct one or more of the steps of the method.

In various embodiments, the microfluidic cartridge comprises two or more sample lanes, each including a sample inlet valve, a bubble removal vent, a thermally actuated pump, a thermally actuated valve, and a PCR reaction zone, wherein the computer readable instructions are configured to independently operate one or more components of each said lane in the system, independently of one another, and for causing a detector to measure fluorescence from the PCR reaction zones.

Sample

In various embodiments, the sample can include a PCR reagent mixture comprising a polymerase enzyme and a plurality of nucleotides. The PCR reagent mixture can be in the form of one or more lyophilized pellets and the steps by which the PCR-ready sample is prepared can involve contacting the PCR pellet with liquid to create a PCR reagent mixture solution. In yet another embodiment, each of the PCR lanes may have dried down or lyophilized ASR reagents preloaded such that the user only needs to input prepared polynucleotide sample into the PCR. In another embodiment, the PCR lanes may have only the application-specific probes and primers premeasured and preloaded, and the user inputs a sample mixed with the PCR reagents.

In various embodiments, the microfluidic network can be configured to couple heat from an external heat source to a sample mixture comprising PCR reagent and neutralized polynucleotide sample under thermal cycling conditions suitable for creating PCR amplicons from the neutralized polynucleotide sample.

In various embodiments, the PCR ready sample can further include a positive control plasmid and a fluorogenic hybridization probe selective for at least a portion of the plasmid. In various embodiments, the PCR-ready sample further includes a sample buffer, and at least one probe that is selective for a polynucleotide sequence, e.g., the polynucleotide sequence that is characteristic of a pathogen selected from the group consisting of gram positive bacteria, gram negative bacteria, yeast, fungi, protozoa, and viruses.

In various embodiments, the microfluidic cartridge can accommodate a negative control polynucleotide, wherein the microfluidic network can be configured to independently carry out PCR on each of a neutralized polynucleotide sample and a negative control polynucleotide with the PCR reagent mixture under thermal cycling conditions suitable for independently creating PCR amplicons of the neutralized polynucleotide sample and PCR amplicons of the negative control polynucleotide. Each lane of a multi-lane cartridge as described herein can perform two reactions because of the presence of two fluorescence detection systems per lane. A variety of combinations of reactions can be performed in the cartridge, such as two sample reactions in one lane, a positive control and a negative control in two other lanes; or a sample reaction and an internal control in one lane and a negative control in a separate lane.

In various embodiments, the sample can include at least one probe that can be selective for a polynucleotide sequence, wherein the steps by which the PCR-ready sample

52

is prepared involve contacting the neutralized polynucleotide sample or a PCR amplicon thereof with the probe. The probe can be a fluorogenic hybridization probe. The fluorogenic hybridization probe can include a polynucleotide sequence coupled to a fluorescent reporter dye and a fluorescence quencher dye. The PCR reagent mixture can further include a positive control plasmid and a plasmid fluorogenic hybridization probe selective for at least a portion of the plasmid and the microfluidic cartridge can be configured to allow independent optical detection of the fluorogenic hybridization probe and the plasmid fluorogenic hybridization probe.

In various embodiments, the probe can be selective for a polynucleotide sequence that is characteristic of an organism, for example any organism that employs deoxyribonucleic acid or ribonucleic acid polynucleotides. Thus, the probe can be selective for any organism. Suitable organisms include mammals (including humans), birds, reptiles, amphibians, fish, domesticated animals, wild animals, extinct organisms, bacteria, fungi, viruses, plants, and the like. The probe can also be selective for components of organisms that employ their own polynucleotides, for example mitochondria. In some embodiments, the probe is selective for microorganisms, for example, organisms used in food production (for example, yeasts employed in fermented products, molds or bacteria employed in cheeses, and the like) or pathogens (e.g., of humans, domesticated or wild mammals, domesticated or wild birds, and the like). In some embodiments, the probe is selective for organisms selected from the group consisting of gram positive bacteria, gram negative bacteria, yeast, fungi, protozoa, and viruses.

In various embodiments, the probe can be selective for a polynucleotide sequence that is characteristic of an organism selected from the group consisting of *Staphylococcus* spp., e.g., *S. epidermidis*, *S. aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Staphylococcus*; *Streptococcus* (e.g., α , β or γ -hemolytic, Group A, B, C, D or G) such as *S. pyogenes*, *S. agalactiae*; *E. faecalis*, *E. durans*, and *E. faecium* (formerly *S. faecalis*, *S. durans*, *S. faecium*); nonenterococcal group *D. streptococci*, e.g., *S. bovis* and *S. equines*, *Streptococci viridans*, e.g., *S. mutans*, *S. sanguis*, *S. salivarius*, *S. mitior*, *A. milleri*, *S. constellates*, *S. intermedius*, and *S. anginosus*; *S. iniae*; *S. pneumoniae*; *Neisseria*, e.g., *N. meningitidis*, *N. gonorrhoeae*, saprophytic *Neisseriasp*; Erysipelothrix, e.g., *E. rhusiopathiae*; *Listeria* spp., e.g., *L. monocytogenes*, rarely *L. ivanovii* and *L. seeligeri*; *Bacillus*, e.g., *B. amhracis*, *B. cereus*, *B. subtilis*, *B. subtilis niger*, *B. thuringiensis*, *Nocardia asteroides*; *Legionella*, e.g., *L. pneumonophila*, *Pneumocystis*, e.g., *P. carinii*; *Enterobacteriaceae* such as *Salmonella*, *Shigella*, *Escherichia* (e.g., *E. coli*, *E. coli*O157:H7); *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella*, *Providencia*, *Yersinia*, and the like, e.g., *Salmonella*, e.g., *S. typhi*, *S. paratyphi* A, B (*S. schoumulleri*), and C (*S. hirschfeldii*), *S. dublin*, *S. choleraesuis*, *S. enteritidis*, *S. typhimurium*, *S. heidelberg*, *S. newport*, *S. infantis*, *S. agona*, *S. montevideo*, and *S. saint-paul*; *Shigella* e.g., subgroups: A, B, C, and D, such as *S. flexneri*, *S. sonnei*, *S. boydii*, *S. dysenteriae*, *Proteus* (*P. mirabilis*, *P. vulgaris*, and *P. myxofaciens*), *Morganella* (*M. morganii*); *Providencia* (*P. rettgeri*, *P. alcalifaciens*, and *P. stuartii*); *Yersinia*, e.g., *Y. pestis*, *Y. enterocolitica*; *Haemophilus*, e.g., *H. influenzae*, *H. parainfluenzae*, *H. aphrophilus*, *H. ducreyi*; *Brucella*, e.g., *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*; *Francisella*, e.g., *F. tularensis*, *Pseudomonas*, e.g., *P. aeruginosa*, *P. paucimobilis*, *P. putida*, *P. fluorescens*, *P. acidovorans*, *Burkholderia* (*Pseudomonas*) *pseudomallei*, *Burkholderia mallei*, *Burk-*

US 10,632,466 B1

53

hoideria cepacia and *Stenotrophomonas maltophilia*, *Campylobacter*, e.g., ; *C. fetus fetus*, *C. jejuni*, *C. pylori* (*Helicobacter pylori*); *Vibrio*, e.g., *V. cholerae*, *V. parahae-*
molyticus, *V. mimicus*, *V. alginolyticus*, *V. hollisae*, *V. vul-*
nificus, and the nonagglutinable vibrios; *Clostridia*, e.g., *C.*
perfringens, *C. tetani*, *C. difficile*, *C. botulinum*; *Actinomy-*
ces, e.g., *A. israelii*; *Bacteroides*, e.g., *B. fragilis*, *B. thetaio-*
taomicron, *B. distasonis*, *B. vulgatus*, *B. ovatus*, *B. caccae*,
 and *B. merdae*; *Prevotella*, e.g., *P. melaninogenica*; genus
Fusobacterium; *Treponema*, e.g., *T. pallidum* subspecies
endemicum, *T. pallidum* subspecies *pertenue*, *T. carateum*,
 and *T. pallidum*, subspecies *pallidum*; genus *Borrelia*, e.g.,
B. burgdorferi; genus *Leptospira*; *Streptobacillus*, e.g., *S.*
moniliformis; *Spirillum*, e.g., *S. minus*; *Mycobacterium*, e.g.,
M. tuberculosis, *M. bovis*, *M. africanum*, *M. avium*, *M.*
intracellulare, *M. kansasii*, *M. xenopi*, *M. marinum*, *M.*
ulcerans, the *M. fortuitum* complex (*M. fortuitum* and *M.*
chelonae), *M. leprae*, *M. asiaticum*, *M. chelonae*, subsp.
abscessus, *M. fallax*, *M. fortuitum*, *M. malmoeense*, *M. shi-*
moidei, *M. simiae*, *M. szulgai*, *M. xenopi*; *Mycoplasma*, e.g.,
M. hominis, *M. orale*, *M. salivarium*, *M. fermentans*, *M.*
pneumoniae, *M. bovis*, *M. tuberculosis*, *M. avium*, *M. lep-*
rae; *Mycoplasma*, e.g., *M. genitalium*; *Ureaplasma*, e.g., *U.*
urealyticum; *Trichomonas*, e.g., *T. vaginalis*; *Cryptococcus*,
 e.g., *C. neoformans*; *Histoplasma*, e.g., *H. capsulatum*;
Candida, e.g., *C. albicans*; *Aspergillus* sp; *Coccidioides*,
 e.g., *C. immitis*; *Blastomyces*, e.g., *B. dermatitidis*; *Para-*
coccidioides, e.g., *P. brasiliensis*; *Penicillium*, e.g., *P. mam-*
effei; *Sporothrix*, e.g., *S. schenckii*; *Rhizopus*, *Rhizomucor*,
Absidia, and *Bastidiobolus*; diseases caused by *Bipolaris*,
Cladophialophora, *Cladosporium*, *Drechslera*, *Exophiala*,
Fonsecaea, *Phialophora*, *Xylohypha*, *Ochroconis*, *Rhin-*
ocladiella, *Scolecobasidium*, and *Wangiella*; *Trichosporon*,
 e.g., *T. beigeli*; *Blastosehiomyces*, e.g., *B. capitatus*; *Plas-*
modium, e.g., *P. falciparum*, *P. vivax*, *P. ovale*, and *P.*
malariae; *Babesia* sp, protozoa of the genus *Trypanosoma*,
 e.g., *T. cruzi*, *Leishmania*, e.g., *L. donovani*, *L. major*, *L.*
tropica, *L. mexicana*, *L. braziliensis*, *L. viannin braziliensis*;
Toxoplasma, e.g., *T. gondii*; *Amoebas* of the genera *Naegle-*
ria or *Acanthamoeba*; *Entamoeba histolytica*; *Giardia lam-*
blia; genus *Cryptosporidium*, e.g., *C. parvum*; *Isospora*
belli; *Cyclospora cayetanensis*, *Ascaris lumbricoides*; *Tri-*
churis trichiura; *Ancylostoma duodenale* or *Necator ameri-*
canus; *Strongyloides stercoralis* *Toxocara*, e.g., *T. canis*, *T.*
cati; *Baylisascaris*, e.g., *B. procyonis*; *Trichinella*, e.g., *T.*
spiralis; *Dracunculus*, e.g., *D. medinensis*; genus *Filarioi-*
dea; *Wuchereria bancrofti*, *Brugia*, e.g., *B. malayi*, nr *B.*
timori; *Onchocerca volvulus*; *Loa loa*; *Dirofilaria immitis*;
 genus *Schistosoma*, e.g., *S. japonicum*, *S. mansoni*, *S.*
mekongi, *S. intercalatum*, *S. haematobium*; *Paragonimus*,
 e.g., *P. westermani*, *P. skrjabini*; *Clonorchis sinensis*; *Fas-*
ciola hepatica; *Opisthorchis* sp; *Fasciolopsis buski*; *Diphyl-*
locularis latum, *Tacnia*, e.g., *T. saginata*, *T. solium*, *Echi-*
nococcus, e.g., *E. granulosus*, *E. multilocularis*;
 Picornaviruses, rhinoviruses echoviruses, coxsackieviruses,
 influenza virus; paramyxoviruses, e.g., types 1, 2, 3, and 4;
 adenoviruses; Herpesviruses, e.g., HSV-1 and HSV-2; vari-
 cella-zoster virus; human T-lymphotropic virus type I and
 type II), Arboviruses and Arenaviruses; *Togaviridae*, *Flavi-*
viridae, *Bunyaviridae*, *Reoviridae*; *Flavivirus*; *Hantavirus*;
 Viral encephalitis (alphaviruses [e.g., Venezuelan equine
 encephalitis, eastern equine encephalitis, western equine
 encephalitis]); Viral hemorrhagic fevers (filoviruses, [e.g.,
 Ebola, Marburg] and arenaviruses (e.g., Lassa, Machupo);
 Smallpox (variola); retroviruses e.g., human immunodef-
 iciency viruses 1 and 2; human papillomavirus (HPV) types
 6, 11, 16, 18, 31, 33, and 35.

54

In various embodiments, the probe can be selective for a
 polynucleotide sequence that is characteristic of an organ-
 isms selected from the group consisting of *Pseudomonas*
aeruginosa, *Proteus mirabilis*, *Klebsiella oxytoca*, *Kleb-*
siella pneumoniae, *Escherichia coli*, *Acinetobacter Bau-*
mannii, *Serratia marcescens*, *Enterobacter aerogenes*,
Enterococcus faecium, vancomycin-resistant *enterococcus*
 (VRE), *Staphylococcus aureus*, methicillin-resistant
Staphylococcus aureus (MRSA), *Streptococcus viridans*,
Listeria monocytogenes, *Enterococcus* spp., *Streptococcus*
 Group B, *Streptococcus* Group C, (*Streptococcus* Group G,
Streptococcus Group F, *Enterococcus faecalis*, *Streptococ-*
cus pneumoniae, *Staphylococcus epidermidis*, *Gardnerella*
vaginalis, *Micrococcus* spp., *Haemophilus influenzae*, *Neis-*
seria gonorrhoeae, *Maraxella catarrhalis*, *Salmonella* spp.,
Chlamydia trachomatis, *Peptostreptococcus productus*,
Peptostreptococcus uuaerabius, *Lactobacillus fermentum*,
Eubacterium lentum, *Candida glabrata*, *Candida albicans*,
Chlamydia spp., *Camplobacter* spp., *Salmonella* spp., small-
 pox (variola major), *Yersinia Pestis*, Herpes Simplex Virus I
 (HSV I), and Herpes Simplex Virus II (HSV II).

In various embodiments, the probe can be selective for a
 polynucleotide sequence that is characteristic of Group B
Streptococcus.

Carrying out PCR on a PCR-ready sample can include
 heating the PCR reagent mixture and the neutralized poly-
 nucleotide sample under thermal cycling conditions suitable
 for creating PCR amplicons from the neutralized polynucle-
 otide sample; contacting the neutralized polynucleotide
 sample or a PCR amplicon thereof with at least one probe
 that is selective for a polynucleotide sequence, indepen-
 dently contacting each of the neutralized polynucleotide
 sample and a negative control polynucleotide with the PCR
 reagent mixture under thermal cycling conditions suitable
 for independently creating PCR amplicons of the neutralized
 polynucleotide sample and PCR amplicons of the negative
 control polynucleotide; and/or contacting the neutralized
 polynucleotide sample or a PCR amplicon thereof and the
 negative control polynucleotide or a PCR amplicon thereof
 with at least one probe that is selective for a polynucleotide
 sequence.

In various embodiments, a method of carrying out PCR on
 a sample can further include one or more of the following
 steps: heating the biological sample in the microfluidic
 cartridge, pressurizing the biological sample in the micro-
 fluidic cartridge at a pressure differential compared to ambi-
 ent pressure of between about 20 kilopascals and 200
 kilopascals, or in some embodiments between about 70
 kilopascals and 110 kilopascals.

In various embodiments, a method of using the apparatus
 described herein can further include one or more of the
 following steps: determining the presence of a polynucle-
 otide sequence in the biological sample, the polynucleotide
 sequence corresponding to the probe, if the probe is detected
 in the neutralized polynucleotide sample or a PCR amplicon
 thereof; determining a contaminated result if the probe is
 detected in the negative control polynucleotide or a PCR
 amplicon thereof; and/or in some embodiments, wherein the
 PCR reagent mixture further comprises a positive control
 plasmid and a plasmid probe selective for at least a portion
 of the plasmid, the method further including determining a
 PCR reaction has occurred if the plasmid probe is detected
 Fluorescence Detection System, Including Lenses and Fil-
 ters, and Multiple Parallel Detection for a Multi-Lane Car-
 tridge

A miniaturized, highly sensitive fluorescence detection
 system can be incorporated for monitoring fluorescence

US 10,632,466 B1

55

from the biochemical reactions that are the basis of nucleic acid amplification methods such as PCR.

Accordingly, another aspect of the apparatus includes a system for monitoring fluorescence from biochemical reactions. The system can be, for example, an optical detector having a light source (for example an LED) that selectively emits light in an absorption band of a fluorescent dye, lenses for focusing the light, and a light detector (for example a photodiode) that selectively detects light in an emission band of the fluorescent dye, wherein the fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof. Alternatively, the optical detector can include a band pass-filtered diode that selectively emits light in the absorption band of the fluorescent dye (a fluorogenic probe) and a bandpass filtered photodiode that selectively detects light in the emission band of the fluorescent dye. For example, the optical detector can be configured to independently detect a plurality of fluorescent dyes having different fluorescent emission spectra, wherein each fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof. For example, the optical detector can be configured to independently detect a plurality of fluorescent dyes at a plurality of different locations of, for example, a microfluidic cartridge, wherein each fluorescent dye corresponds to a fluorescent polynucleotide probe or a fragment thereof.

In some embodiments, a given detector for use with the apparatus described herein is capable of detecting a fluorescence signal from nanoliter scale PCR reactions. Advantageously, the detector is formed from inexpensive components, having no moving parts. The detector is also configured to mate with a microfluidic cartridge as further described herein, and is also preferably part of a pressure application system, such as a sliding lid, that keeps the cartridge in place. The detector further has potential for 2 or 3 color detection and is controlled by software, preferably custom software, configured to sample information from the detector.

FIGS. 57-59 depict an embodiment of a highly sensitive fluorescence detection system including light emitting diodes (LED's), photodiodes, and filters/lenses for monitoring, in real-time, one or more fluorescent signals emanating from the microfluidic cartridge. The embodiment in FIGS. 57-59 has a two-color detection system having a modular design that mates with a single lane microfluidic cartridge. The detector comprises two LED's (blue and red, respectively) and two photodiodes. The two LED's are configured to transmit a beam of focused light on to a particular region of the cartridge. The two photodiodes are configured to receive light that is emitted from the region of the cartridge. One photodiode is configured to detect emitted red light, and the other photodiode is configured to detect emitted blue light.

FIGS. 60 and 61 show an exemplary read-head comprising a multiplexed 2 color detection system, such as multiple instances of a detection system shown in FIGS. 57-59, that is configured to mate with a multi-lane microfluidic cartridge. FIG. 60 shows a view of the exterior of a multiplexed read-head. FIG. 61 is an exploded view that shows how various detectors are configured within an exemplary multiplexed read head, and in communication with an electronic circuit board.

The module in FIGS. 60 and 61 is configured to detect fluorescence from each lane of a 12-lane cartridge, and therefore comprises 24 independently controllable detectors, arranged as 12 pairs of identical detection elements. Each pair of elements is then capable of dual-color detection of a

56

pre-determined set of fluorescent probes. It would be understood by one of ordinary skill in the art that other numbers of pairs of detectors are consistent with the apparatus described herein. For example, 4, 6, 8, 10, 16, 20, 24, 25, 30, 32, 36, 40, and 48 pairs are also consistent and can be configured according to methods and criteria understood by one of ordinary skill in the art.

Exemplary Optics Assembly

In an exemplary embodiment, the optical chassis/pressure assembly is housed in an enclosure (made of plastic in certain embodiments) that can be positioned to cover multi-lane microfluidic cartridge. The enclosure can optionally have a handle that can be easily grasped by a user, and is guided for smooth and easy pushing and pulling. The handle may also serve as a pressure-locking device. The enclosure's horizontal position is sensed in both the all-open and in the all-forward position, and reported to controlling software. The enclosure and optical chassis pressure assembly registers with a heater cassette module positioned underneath a microfluidic cartridge to within 0.010". A close fit is important for proper heater/cartridge interface connections. The enclosure assembly does not degrade in performance over a life of 10,000 cycles, where a cycle is defined as: beginning with the slider in the back position, and sliding forward then locking the handle down on a cartridge, unlocking the handle and returning it to the original back position. All optical path parts should be non-reflective (anodized, painted, molded, etc.) and do not lose this feature for 10,000 cycles. The optics unit is unaffected by a light intensity of $\leq 9,000$ foot-candles from a source placed 12" from the instrument at angles where light penetration is most likely to occur. No degradation of performance is measured at the photo-detector after 10,000 cycles.

When fabricating a detector assembly, a single channel is made that houses two LED sources (blue and amber) and two additional channels that house one photodiode detector each (four total bored holes). The two paired channels (source and detector) are oriented 43° from each other, measured from the optical axis and are in-line with the other paired channels that are at the same 43° orientation. The holes bored in the optical chassis contain fibers and lenses with appropriate spacers, the specifications of which are further described herein. The LED's are held in place to prevent movement as the mechanical alignment is important for good source illumination. The LED's are preferably twisted until the two "hot spots" are aligned with the reading channels on the cartridge. This position must be maintained until the LED's cannot be moved. The optical chassis can be made of aluminum and be black anodized. The bottom pressure surface of the optical chassis is flat to $\pm 0.001"$ across the entire surface. The optical chassis is center-balanced such that the center of the optical chassis is close to the center of the reagent cartridge. The pressure assembly (bottom of the optical chassis) provides uniform pressure of a minimum of 1 psi across all heater sections of the reagent cartridge. The optical assembly can be moved away from the reagent cartridge area for cartridge removal and placement. Appropriate grounding of the optical chassis is preferred to prevent spurious signals to emanate to the optical PCH.

The LED light sources (amber and blue) are incident on a microfluidic cartridge through a band pass filter and a focusing lens. These LED light sources have a minimum output of 2800 millicandles (blue) and 5600 millicandles (Green), and the center wavelengths are 470 (blue) and 575 (amber) nanometers, with a half band width of no more than 75 nanometers.

US 10,632,466 B1

57

The LED light excites at least one fluorescent molecule (initially attached to an oligonucleotide probe) in a single chamber on a cartridge, causing it to fluoresce. This fluorescence will normally be efficiently blocked by a closely spaced quencher molecule. DNA amplification via TAQ enzyme will separate the fluorescent and quenching molecules from the oligonucleotide probe, disabling the quenching. DNA amplification will only occur if the probe's target molecule (a DNA sequence) is present in the sample chamber. Fluorescence occurs when a certain wavelength strikes the target molecule. The emitted light is not the same as the incident light. Blue incident light is blocked from the detector by the green only emission filter. Green incident light similarly is blocked from the detector by the yellow emission filter. The fluorescent light is captured and travels via a pathway into a focusing lens, through a filter and onto a very sensitive photodiode. The amount of light detected increases as the amount of the DNA amplification increases. The signal will vary with fluorescent dye used, but background noise should be less than 1 mV peak-to-peak. The photo-detector, which can be permanently mounted to the optical chassis in a fixed position, should be stable for 5 years or 10,000 cycles, and should be sensitive to extremely low light levels, and have a dark value of no more than 60 mV. Additionally, the photo-detector must be commercially available for at least 10 years. The lenses are Plano-convex (6 mm detector, and 12 mm source focal length) with the flat side toward the test cartridge on both lenses. The filters should remain stable over normal operating humidity and temperature ranges.

The filters, e.g., supplied by Omega Optical (Brattleboro, Vt. 05301), are a substrate of optical glass with a surface quality of PF/F P per Mil-C-48497A. The individual filters have a diameter of 6.0 ± 0.1 mm, a thickness of 6.0 ± 0.1 mm, and the AOI and $\frac{1}{2}$ cone AOI is 0 degrees and ± 8 degrees, respectively. The clear aperture is ≥ 4 mm diameter and the edge treatment is blackened prior to mounting in a black, anodized metal ring. The FITC exciter filters is supplied by, e.g., Omega Optical (PN 481AF30-RFD-EXC). They have a cut-off frequency of 466 ± 4 nm and a cut-on frequency of 496 ± 4 nm. Transmission is $\geq 65\%$ peak and blocking is: $\geq OD8$ in theory from 503 to 580 nm, $\geq OD5$ from 501-650 nm, $\geq OD4$ avg over 651-1000 nm, and $\geq OD4$ UV-439 nm. The FITC emitter filters is supplied by, e.g., Omega Optical (PN 534AF40-RED-EM). They will have a cut-off frequency of 514 ± 2 nm and a cut-on frequency of 554 ± 4 nm. Transmission is $\geq 70\%$ peak and blocking is: $\geq OD8$ in theory from 400 to 504 nm, $\geq OD5$ UV-507 nm, and $\geq OD4$ avg, 593-765 nm. The amber exciter filters are supplied by, e.g., Omega Optical (PN 582AF25-RED-EXC). They have a cut-off frequency of 594 ± 5 nm and a cut-on frequency of 569 ± 5 nm. Transmission is $\geq 70\%$ peak and blocking is $\geq OD8$ in theory from 600 to 700 nm, $\geq OD5$ 600-900 nm, and $\geq OD4$ UV-548 nm. The amber emitter filters are supplied by, e.g., Omega Optical (PN 627AF30-RED-EM). They have a cut-off frequency of 642 ± 5 nm and a cut-on frequency of 612 ± 5 nm. Transmission is $\geq 70\%$ peak and blocking is: $\geq OD8$ in theory from 550 to 600 nm, $\geq OD5$ UV-605 nm, and $\geq OD5$ avg. 667-900 nm. The spacers should be inert and temperature stable throughout the entire operating range and should maintain the filters in strict position and alignment. The epoxy used should have optically black and opaque material and dry solid with no tacky residue. Additionally, it should have temperature and moisture stability, exert no pressure on the held components, and should mount the PCB in such a way that it is fixed and stable with no chances of rotation or vertical height changes.

58

50% of illumination shall fall on the sample plane within an area 0.1" (2.5 mm) wide by 0.3" (7.5 mm) along axis of the detection channel. Fluorescence of the control chip should not change more than 0.5% of the measured signal per 0.001" of height though a region ± 0.010 from the nominal height of the control chip.

An exemplary optics board is shown in FIG. 62, and is used to detect and amplify the fluorescent signature of a successful chemical reaction on a micro-fluidic cartridge, and controls the intensity of LED's using pulse-width modulation (PWM) to illuminate the cartridge sample over up to four channels, each with two color options. Additionally, it receives instructions and sends results data back over an LVDS (low-voltage differential signaling) SPI (serial peripheral interface). The power board systems include: a +12V input; and +3.3V, +3.6V, +5V, and -5V outputs, configured as follows: the +3.3V output contains a linear regulator, is used to power the LVDS interface, should maintain a $\pm 5\%$ accuracy, and supply an output current of 0.35 A; the ± 3.6 V output contains a linear regulator, is used to power the MSP430, should maintain a $\pm 5\%$ accuracy, and supply an output current of 0.35 A; the +5V output contains a linear regulator, is used to power the plus rail for op-amps, should maintain a $\pm 5\%$ accuracy, and supply an output current of 0.35 A; the -5V output receives its power from the +5V supply, is used to power the minus rail the op-amps and for the photo-detector bias, should maintain a $\pm 1\%$ voltage accuracy, and supply an output current of 6.25 mA $\pm 10\%$. Additionally, the power board has an 80 ohm source resistance, and the main board software can enable/disable the regulator outputs.

The main board interface uses a single channel of the LVDS standard to communicate between boards. This takes place using SPI signaling over the LVDS interface which is connected to the main SPI port of the control processor. The interface also contains a serial port for in-system programming.

The exemplary optical detection system of FIG. 62 consists of a control processor, LED drivers, and a photo-detection system. In the exemplary embodiment the control processor is a T1 MSP430F1611 consisting of a dual SPI (one for main board interface and one for ADC interface) and extended SRAM for data storage. It has the functions of power monitoring, PWM LED control, and SPI linking to the ADC and main board. The LED drivers contain NPN transistor switches, are connected to the PWM outputs of the control processor, can sink 10 mA @12 V per LED (80 mA total), and are single channel with 2 LEDs (one of each color) connected to each. The photo-detection system has two channels and consists of a photo-detector, high-sensitivity photo-diode detector, high gain current to voltage converter, unity gain voltage inverting amplifier, and an ADC. Additionally it contains a 16 channel Sigma-delta (only utilizing the first 8 channels) which is connected to the second SPI port of the control processor. It would be understood by one of ordinary skill in the art that other choices and combinations of elements can be brought together to make a functioning detection system consistent with the description herein.

Additional Advantages and Features of the Technology Herein

The use of a disposable process chamber, having surface coating and material properties to allow low volume, and open tube heated release to maximize sample concentration in lowest volume possible.

US 10,632,466 B1

59

The integrated magnetic heat separator that allows multiple samples to be heated independently but separated using a single moveable magnet platform.

A reader/bay design that allows easy placement of microfluidic cartridge and multiple sample pipetting of liquid using a robotic dispenser in one position; relative displacement to another location and pressure application for subsequent rapid heat incubation steps and optical detection. The bottom surface of the cartridge mates with the heating surface. Furthermore, it is typically easier to move a cartridge and heater in and out of position than a detector.

A moveable readhead design for fluorescence detection from microfluidic PCR channels,

Aspects of the holder, such as a unitized disposable strip, that include the presence of sealed lyophilized reagents as well as liquids sealed in close proximity, which is normally hard to achieve. The laminates deployed herein make storage easier.

The holder permits snapping of multiple ASR tubes, and associated liquid dispensing processes that minimizes cross-sample contamination but multiple PCR preparations to be performed from a single clinical sample.

Software features allow a user to either get results from all 24 samples as quickly as possible or the first 12 samples as quickly as possible and the next 12 later.

The preparatory and diagnostic instruments described herein enables different sample types (such as blood, urine, swab, etc.) to be all processed at the same time even though each may require different temperatures, times or chemical reagents. This is achieved in part by using individualized but compatible holders.

Automatic feeding of microfluidic cartridges into a PCR reader via a cartridge autoloader saves a user time and leads to increased efficiency of overall operation.

Piercing through foil over a liquid tube and reliable way of picking up liquid.

A moveable read-head that has the pumps, sensors (pipette detection, force sensing), sample identification verifier, etc., moving with it, and therefore minimizes the number of control lines that move across the instrument during use.

Accurate and rapid alignment of pipette tips with cartridge inlet holes using a motorized alignment plate.

EXAMPLES

Example 1: Reagent Holder

An exemplary reagent holder consistent with the description herein has the following dimensions and capacities 180 mm longx22 mm widex100 mm tall;

Made from Polypropylene.

One snapped-in low binding 1.7 ml tube that functions as a process tube.

3 built-in tubes that function as receptacles for reagents, as follows:

One tube containing 200-1000 μ l of wash buffer (0.1 mM Tris, pH 8).

One tube containing 200-1000 μ l of release solution (40 mM NaOH).

One tube containing 200-1000 μ l of neutralization solution (330 mM Tris, pH 8.0).

One built-in tube that functions as a waste chamber (will hold ~4 ml of liquid waste).

3 receptacles to accept containers for solid reagents.

Snap-in 0.3 ml or 0.65 ml OCR tubes (which are

60

typically stored separately from the reagent holder) are placed in each of these locations, and contain, respectively:

lyophilized sample preparation reagents (lysis enzyme mix and magnetic affinity beads).

First lyophilized PCR master mix, probes and primers for a first target analyte detection.

Second lyophilized PCR master mix, probes and primers for a second target analyte detection (only offered in select cases, such as detection of Chlamydia and Gonorrhea from urine).

4 pipette tips located in 4 respective sockets.

Pipette tip Sheath: The pipette tips have a sheath/drip tray underneath to help capture any drip from the pipette tips after being used, and also to prevent unwanted contamination of the instrument.

Handle and Flex-Lock allows easy insertion, removal, and positive location of strip in rack.

One or more labels: positioned upward facing to facilitate ease of reading by eye and/or, e.g., a bar-code reader, the one or more labels containing human and machine readable information pertaining to the analysis to be performed.

It is to be understood that these dimensions are exemplary. However, it is particularly desirable to ensure that a holder does not exceed these dimensions so that a rack and an apparatus that accommodates the reagent holder(s) does not become inconveniently large, and can be suitably situated in a laboratory, e.g., on a bench-top.

Example 2: Disposable Reagent Holder Manufacturing

Simple fixtures can be designed and machined to enable handling and processing of multiple strips. There are five steps that can be performed to produce this component. The disposable reagent holder will be placed in a fixture and filled with liquids using manual/electric-multiple pipetting. Immediately after dispensing all liquids into the strip, foil will be heat sealed to the plastic using exemplary heat seal equipment (Hix FH-3000-D Fiat Head Press) and the foil trimmed as required. After heat sealing liquids on board, all pellets in tubes can be snapped into the strip, pipette tips can be inserted in their respective sockets, and a barcode label can be affixed. Desiccant packs can be placed into the blow molded or thermoformed rack designed to house 12 holders. Twelve disposable strips will be loaded into the rack and then sealed with foil. The sealed bag will be placed into a carton and labeled for shipping.

Example 3: Foil-sealing of Buffer Containing Reagent Tubes

Tubes containing buffers have to be sealed with high moisture vapor barrier materials in order to retain the liquid over a long period of time. Disposable holders may need to have a shelf life of 1-2 years, and as such, they should not lose more than say 10-15% of the liquid volume over the time period, to maintain required volume of liquid, and to maintain the concentration of various molecules present in the solution. Moreover, the materials used for construction of the tube as well as the sealing laminate should not react with the liquid buffer. Special plastic laminates may provide the moisture barrier but they may have to be very thick (more than 300 μ m thick), causing the piercing force to go up tremendously, or of special, expensive polymer (such as Aclar). Aluminum foils, even a thin foil of a few hundred

US 10,632,466 B1

61

angstrom provides an effective moisture barrier but bare aluminum reacts with some liquid buffers, such as sodium hydroxide, even an aluminum foil with a sprayed coating of a mm-reactive polymer may not be able to withstand the corrosive vapors over a long time. They may react through tiny pin holes present in the coating and may fail as a barrier over time.

For these reasons, aluminum foils with a laminate structure have been identified as a suitable barrier, exemplary properties of which are described below:

1. Sealing

Heat seals to unitized polypropylene strip (sealing temp~170-180° C.) No wrinkling, cracking and crazing of the foil after sealing

2. Moisture Vapor Transmission Rate (MVTR)

Loss of less than 10% liquid (20 microliters from a volume of 200 microliter) for a period of 1 year stored at ambient temperature and pressure, (effective area of transport is ~63 mm²): Approximate MVTR~0.8 cc/m²/day

3. Chemistry

Ability to not react with 40 mM Sodium Hydroxide (pH<12.6): foil should have a plastic laminate at least 15 microns thick closer to the sealed fluid

Ability to not react with other buffers containing mild detergents

4. Puncture

Ability to puncture using a p1000 pipette with a force less than 3 lb

Before puncturing, a fully supported membrane 8 mm in diameter will not stretch more than 5 mm in the orthogonal direction

After puncturing, the foil should not seal the pipette tip around the circumference of the pipette.

5. Other Features

Pin-hole free

No bubbles in case of multi-laminate structures.

Example 4: Mechanism of Piercing through a Plasticized Laminate and Withdrawing Liquid Buffer

The aluminum laminate containing a plastic film described elsewhere herein serves well for not reacting with corrosive reagents such as buffers containing NaOH, and having the favorable properties of pierceability and acting as a moisture barrier. However, it presents some additional difficulties during piercing. The aluminum foil tends to burst into an irregular polygonal pattern bigger than the diameter of the pipette, whereas the plastic film tends to wrap around the pipette tip with minimal gap between the pipette and the plastic film. The diameter of the hole in the plastic film is similar to the maximum diameter of the pipette that had crossed through the laminate. This wrapping of the pipette causes difficulty in dispensing and pipetting operations unless there is a vent hole allowing pressures to equilibrate between outside of the tube and the air inside of the tube.

A strategy for successful pipetting of fluid is as follows:

1. Pierce through the laminate structure and have the pipette go close to the bottom of the reagent tube so that the hole created in the laminate is almost as big as the maximum diameter of the pipette (e.g., ~6 mm for a p1000 pipette)

2. Withdraw the pipette up a short distance so that a small annular vent hole is left between the pipette and the laminate. The p1000 pipette has a smallest outer diameter of 1 mm and maximum outer diameter of 6 mm and

62

the conical section of the pipette is about 28 mm long. A vent hole thickness of a hundred microns is enough to create a reliable vent hole. This corresponds to the pipette inserted to a diameter of 5.8 mm, leaving an annulus of 0.1 mm around it.

3. Withdraw fluid from the tube. Note that the tube is designed to hold more fluid than is necessary to withdraw from it for a sample preparation procedure.

Example 5: Foil Piercing and Dissolution of Lyophilized Reagents:

The containers of lyophilized reagents provided in conjunction with a holder as described herein are typically sealed by a non-plasticized aluminum foil (i.e., not a laminate as is used to seal the reagent tubes). Aluminum foil bursts into an irregular polygonal pattern when pierced through a pipette and leaves an air vent even though the pipette is moved to the bottom of the tube. In order to save on reagents, it is desirable to dissolve the reagents and maximize the amount withdrawn from the tube. To accomplish this, a star-ridged (stellated) pattern is placed at the bottom of the container to maximize liquid volume withdrawn, and flow velocity in between the ridges.

Exemplary steps for dissolving and withdrawing fluid are as follows:

1. Pierce through the pipette and dispense the fluid away from the lyophilized material. If the pipette goes below the level of the lyophilized material, it will go into the pipette and may cause jamming of the liquid flow out of the pipette.
2. Let the lyophilized material dissolve for a few seconds.
3. Move pipette down touching the ridged-bottom of the tube.
4. Perform an adequate number of suck and spit operations (4-10) to thoroughly mix the reagents with the liquid buffer.
5. Withdraw all the reagents and move pipette to dispense it into the next processing tube.

Example 6: Material and Surface Property of the Lysis Tube

The material, surface properties, surface finish has a profound impact on the sensitivity of the assay performed. In clinical applications, DNA/RNA as low as 50 copies/sample (~1ml volume) need to be positively detected in a background of billions of other molecules, some of which strongly inhibit PCR. In order to achieve these high level of sensitivities, the surface of the reaction tube as well as the material of the surface has to be chosen to have minimal binding of polynucleotides. During the creation of the injection molding tool to create these plastic tubes, the inherent surfaces created by machining may have large surface area due to cutting marks as large as tens of microns of peaks and valleys. These surfaces have to be polished to SPI A2/A2 finish (mirror finish) to remove the microscopic surface irregularities. Moreover, the presence of these microscopic valleys will trap magnetic beads (0.5-2 μ) at unintended places and cause irregular performance. In addition to actual surface roughness, the surface hydrophobicity/surface molecules present may cause polynucleotides to stick at unintended places and reduce sensitivity of the overall test. In addition to the base material uses, such as homogenous polypropylene and other polymers, specific materials used during the molding of these tubes, such as mold release

US 10,632,466 B1

63

compounds or any additives to aid in the fabrication can have a profound impact on the performance of the reactions.

Example 7: Liquid Dispensing Head

Referring to FIGS. 18, 19A-C, and 63, an exemplary liquid dispenser is attached to a gantry, and receives instructions via electrical cable 1702. Barcode scanner 1701 is mounted on one face of the liquid dispenser. The gantry is mounted on a horizontal rail 1700 to provide movement in the x-direction. Not shown is an orthogonally disposed rail to provide movement in the y-direction. The liquid dispenser comprises a computer controlled motorized pump 1800 connected to fluid distribution manifold 1802 with related computer controlled valving 1801 and a 4-up pipette with individually sprung heads 1803. The fluid distribution manifold has nine Lee Co. solenoid valves 1801 that control the flow of air through the pipette tips: two valves for each pipette, and an additional valve to vent the pump. Barcode reader 1701 enables positive detection of sample tubes, reagent disposables and microfluidic cartridges. The scanner is mounted to the z-axis so that it can be positioned to read the sample tube, strip, and cartridge barcodes.

Example 8: Integrated Heater/Separator

In FIG. 64 an exemplary integrated magnetic separator and heater assembly are shown. Magnetic separator 1400 and heater assembly 1401 were fabricated comprising twelve heat blocks aligned parallel to one another. Each heat block 1403 is made from aluminum, and has an L-shaped configuration having a U-shaped inlet for accepting a process chamber 1402. Each heat block 1403 is secured and connected by a metal strip 1408 and screws 1407. Magnet 1404 is a rectangular block Neodymium (or other permanent rare earth materials, K&J Magnetics, Forcefield Magnetics) disposed behind each heat block 1403 and mounted on a supporting member. Gears 1406 communicate rotational energy from a motor (not shown) to cause the motorized shaft 1405 to raise and lower magnet 1404 relative to each heat block. The motor is computer-controlled to move the magnet at speeds of 1-20 mm/s. The device further comprises a printed circuit board (PCB) 1409 configured to cause the heater assembly to apply heat independently to each process chamber 1402 upon receipt of appropriate instructions. In the exemplary embodiment, the device also comprises a temperature sensor and a power resistor in conjunction with each heater block.

Example 9: Exemplary Software

Exemplary software accompanying use of the apparatus herein can include two broad parts-user interface and device firmware. The user interface software can allow for aspects of interaction with the user such as - entering patient/sample information, monitoring test progress, error warnings, printing test results, uploading of results to databases and updating software. The device firmware can be the low level software that actually runs the test. The firmware can have a generic portion that can be test independent and a portion specific to the test being performed. The test specific portion ("protocol") can specify the microfluidic operations and their order to accomplish the test.

FIGS. 65A and 65B show screen captures from the programming interface and real time heat sensor and optical

64

detector monitoring. This real time device performance monitoring is for testing purposes; not visible to the user in the final configuration.

User Interface:

A medical grade LCD and touch screen assembly can serve as the user interface via a graphical user interface providing easy operating and minor troubleshooting instructions. The LCD and touch screen have been specified to ensure compatibility of all surfaces with common cleaning agents. A barcode scanner integrated with the analyzer can be configured to scan the barcode off the cartridge (specifying cartridge type, lot#, expiry date) and if available the patient and user ID from one or more sample tubes.

Example 10: Exemplary Preparatory Apparatus

This product is an instrument that enables 24 clinical samples to be automatically processed to produce purified nucleic acid (DNA or RNA) in about half an hour (FIG. 66). Purified nucleic acid may be processed in a separate amplification-detection machine to detect the presence of certain target nucleic acids. Samples are processed in a unitized disposable strip, preloaded with sample preparation chemistries and final purified nucleic acids are dispensed into PCR tubes. Fluid handling is enabled by a pipetting head moved by a xyz gantry, (FIG. 67).

The System has the following sub-systems:

- Two sample processing racks, each rack processes up to 12 clinical samples in unitized disposable strips
- Magnetic separator-cum-tube heater assembly (24 heating stations)
- A four-probe liquid dispensing head
- 3-axis gantry to move the pipette head
- Peltier-cooled pcr-tube holding station to receive the purified DNA/RNA
- Control electronics
- Barcode reader

Operation: The user will get a work list for each sample, whether they want to extract DNA or RNA for each clinical sample. The sample tubes are placed on the rack and for each sample type (DNA or RNA), the user slides in a unitized reagent disposable (DNA or RNA processing) into corresponding lane of the rack. The unitized disposable (holder) will have all the sample prep reagents, process tubes as well as disposable pipettes already prepackaged in it. Once all disposables are loaded into the rack, the rack is placed in its location on the instrument. Open pcr tubes are placed in the peltier cooled tube holder where the final purified nucleic acid will be dispensed. The user then closes the door of the instrument and then starts the sample processing using the GUI (Graphical User Interface).

The instrument checks functionality of all subsystems and then reads the barcode of the sample tubes and the unitized reagent disposable. Any mismatch with a pre-existing work list is determined and errors are flagged, if necessary. The instrument then goes through a series of liquid processing, heating, magnetic separations to complete the sample preparation steps for each of the clinical sample and outputs the purified nucleic acid into the PCR tube. The basic steps involved in each sample processing are sample lysis, nucleic acid capture into magnetic affinity beads, washing of the magnetic beads to remove impurities, releasing the nucleic acid from the magnetic beads, neutralizing the released DNA and the dispensing into the final PCR tube. These tubes

US 10,632,466 B1

65

are maintained at 4° C. until all samples are processed and user takes away the tube for downstream processing of the nucleic acids.

Example 11: Exemplary Diagnostic Apparatus

The apparatus, in combination with the associated consumables, automatically performs all aspects of nucleic acid testing, including sample preparation, amplification, and detection for up to 48 samples per hour with the first 24 results available in less than an hour. The system is easy to use. An operator simply aliquots a portion of the patient sample into a dedicated tube that contains pre-packaged buffer. The operator places the dedicated tubes into positions on a sample rack. The operator then loads a disposable plastic reagent strip for the appropriate test in the rack. The only other consumable used in the apparatus are microfluidic PCR cartridges for conducting amplification and detection; each cartridge is capable of performing up to twelve PCR tests and two cartridges can be loaded into the analyzer at once. Should the apparatus require a new PCR cartridge, the analyzer will prompt the operator to load the cartridge. The analyzer will then prompt the operator to close the lid to initiate testing. All consumables and sample tubes are bar-coded for positive sample identification.

Sample lysis and DNA preparation, which will require approximately half an hour for a full run of 24 samples, is automatically performed by the analyzer's robotic and liquid handling components using protocols and reagents located in unitized, disposable plastic strips. The apparatus then automatically mixes the sample and PCR reagents, and injects the mixture into a cartridge that will be automatically processed by an integrated PCR machine. Rapid, real time PCR and detection requires less than 20 minutes. Results, which will be automatically available upon completion of PCR, are displayed on the instruments touch screen, printed or sent to the hospital information system, as specified by the user (or the user's supervisor).

Each instrument can process up to 24 samples at a time with a total throughput of 48 samples per hour after the first run. The analyzer is slightly less than 1 m wide and fits easily on a standard lab bench. All operations of the unit can be directed using the included barcode wand and touch screen. The analyzer can be interfaced with lab information systems, hospital networks, PCs, printers or keyboards through four USB interfaces and an Ethernet port.

The apparatus has the following characteristics.

Sensitivity the apparatus will have a limit of detection of ~50 copies of DNA or RNA; (and may have a limit of detection as low as 25-30 copies of DNA/RNA).

Cost per Test: Due to the miniaturized, simplified nature of HandyLab reagents, cartridge and other consumables, the cost of goods per test will be relatively low and very competitive.

Automation: By contrast with current "automated" NAT systems, which all require some degree of reasonably extensive technologist interaction with the system, through the use of unitized tests and full integration of sample extraction, preparation, amplification and detection, the apparatus herein will offer a higher level of automation, and corresponding reduction in technologist time and required skill level, thereby favorably impacting overall labor costs.

Throughput: Throughput is defined as how many tests a system can conduct in a given amount of time. The apparatus will be capable of running 45 tests per hour, on average.

Time to First Result: In a hospital environment, time to first result is an especially important consideration. The

66

apparatus will produce the first 24 results in less than an hour and an additional 24 results every half hour thereafter.

Random Access and STAT: Random access is the ability to run a variety of tests together in a single run and place samples in unassigned locations on the analyzer. Also, with chemistry and immunoassay systems, it is desirable to be able to add tests after a run has started. This is often referred to as "true random access" since the user is provided complete flexibility with regard to what tests can be run where on an analyzer and when a new sample can be added to a run. A STAT is a sample that requires as rapid a result as possible, and therefore is given priority in the testing cue on the analyzer. Today, essentially all chemistry and immunoassay analyzers are true random access and offer STAT capabilities. For NAT, however, very few systems offer any random access or STAT capabilities. The instrument herein will provide random access and STAT capabilities.

Menu: The number and type of tests available for the analyzer is a very important factor in choosing systems. The apparatus herein deploys a launch menu strategy that involves a mix of high volume, "standard" nucleic acid tests combined with novel, high value tests.

The apparatus enables 24 clinical samples to be automatically processed to purify nucleic acid, mix the purified DNA/RNA with PCR reagents and perform real-time PCR in microfluidic cartridge to provide sample to results in an hour. The exemplary apparatus has two PCR readers, each capable of running a 12 lane microfluidic cartridge using an optical system that has dedicated two-color optical detection system. FIG. 68, FIG. 69.

The apparatus has the following sub-systems:

- Two sample processing racks, each rack processes up to 12 clinical samples in unitized disposable strips
- Magnetic separator-cum-tube heater assembly (24 heating stations)

- A four-probe liquid dispensing head

- 3-axis gantry to move the pipette head

- Two PCR amplification-detection station, each capable of running a 12-lane microfluidic cartridge and dedicated 2-color optical detection system for each PCR lane.

- Control electronics

- Barcode reader

Pictures of exterior (face on) and interior are at FIGS. 70, 71, respectively.

Operation: The user will get a work list for each sample, whether they want to detect certain target analyte (such as GBS, Chlamydia, Gonorrhea, HSV) for each clinical sample. The sample tubes are placed on the rack and for each sample, the user slides in a unitized reagent disposable (analyte specific) into corresponding lane of the rack. The unitized disposable will have all the sample prep reagents, PCR reagents, process tubes as well as disposable pipettes already prepackaged in it. Once all disposables are loaded into the rack, the rack is placed in its location on the instrument. The user then places two 12-lane microfluidic PCR cartridges in the two trays of the PCR reader. The user then closes the door of the instrument and then starts the sample processing using the GUI (Graphical User Interface).

The instrument checks functionality of all subsystems and then reads the barcode of the sample tubes, the unitized reagent disposables and the microfluidic cartridges. Any mismatch with a pre-existing work list is determined and errors are flagged, if necessary. The instrument then goes through a series of liquid processing, heating, magnetic separation to complete the sample preparation steps for the each of the clinical sample, mixes the purified nucleic acid with PCR reagents and dispenses the final mix into a lane of

US 10,632,466 B1

67

the microfluidic cartridges. After a microfluidic cartridge is loaded with the final PCR mix, the cartridge tray moves and aligns the cartridge in the reader and the optical detection system presses the cartridge against a microfluidic PCR heater surface. On-chip valves are actuated to close the reaction mix and then thermocycling is started to initiate the PCR reaction. At each cycle of PCR (up to 45 cycles), fluorescence from each PCR lane is detected by the optical detection system (2-colors per PCR lane) and final result is determined based on the threshold cycle (Ct).

The sample preparation steps for 24 samples are performed in about 40 minutes and the PCR reaction in about 20 minutes.

Sample Reader:

The Reader performs function testing of up to twelve properly prepared patient samples by PCR process (real-time PCR) when used in conjunction with HandyLab microfluidic (test) cartridges. Each unit will employ two Reader Modules for a total of up to twenty four tests. (FIGS. 72A and 72B) Operation of the Reader is designed for minimal customer interaction, requiring the loading and unloading of test cartridges only. During the "Load Disposables" sequence, the Reader will present a motor actuated tray for installation of the disposable cartridge. Sliding a small knob located in the front of the tray, a spring loaded protective cover will raise allowing the test cartridge to be nested properly in place. The cover is then lowered until the knob self-locks into the tray frame, securing the cartridge and preventing movement during the sample loading sequence.

Once the prepared samples have been dispensed via pipettes into the test cartridge, the tray will retract into the Reader, accurately positioning the test cartridge beneath the chassis of the optical assembly. The optical assembly will then be lowered by a captured screw driven stepper motor until contact is made with the test cartridge. At this point the test cartridge is located $\frac{1}{8}$ above the target location on the heater assembly. As downward motion continues the test cartridge and its holder within the tray compress springs on the tray frame (these are used later to return the cartridge to its normal position and able to clear the encapsulated wire bonds located on the heater assembly during tray operation). Movement of the test cartridge and optical assembly is complete once contact with the heater assembly is made and a minimum of 2 psi is obtained across the two-thirds of the cartridge area about the PCR channels and their controlling gates. At this point the testing of the cartridge is performed using the heater assembly, measured with onboard optics, and controlled via software and electronics much in the same manner as currently operated on similar HandyLab instruments.

Once the functional testing is complete the main motor raises the optic assembly, releasing pressure on the test cartridge to return to its normal position. When commanded, the tray motor operating in a rack-and pinion manner, presents the tray to the customer for cartridge removal and disposal. When the tray is in the extended position it is suspended above a support block located on the apparatus chassis. This block prevents the cartridge from sliding through the holder in the tray during loading and acts as a support while samples are pipetted into the disposable cartridge. Also provided in this support block is an assist lever to lift and grasp the disposable cartridge during removal. All components of the tray as well as support block and cartridge lift assist are removable by the customer, without tools, for cleaning and reinstalled easily.

68

Microfluidic PCR Heater Module:

The microfluidic PCR heater module comprises a glass wafer with photolithographically defined microheaters and sensors to accurately provide heat for actuation of valves and performing thermocycling required to perform a real-time PCR reaction. The wafer surface has dedicated individually controlled heating zones for each of the PCR lanes in the microfluidic cartridge. For a 12-up cartridge, there are 12 PCR zones and the 24-up cartridge, there are 24 PCR heating zones. The individual heaters and sensors are electrically connected to a Printed circuit board using gold or aluminum wire bonds. A thermally compliant encapsulant provides physical protection the wirebonds. While the present device is made on glass wafer, heaters can be fabricated on Si-on-Glass wafers and other polymeric substrates. Each substrate can have provide specific advantages related to its thermal and mechanical properties. Besides using photolithography process, such heating substrates can also be assembled using off-the-shelf electronic components such as power resistors, peltiers, transistors, maintaining the upper heating surface of each of the component to be at the same level to provide heating to a microfluidic cartridge. Temperature calibration values for each temperature sensor may be stored in a EEPROM or other memory devices co-located in the heater PCBoard.

12-Lane Cartridge:

This 12 channel cartridge is the same basic design that is described in U.S. provisional patent application Ser. No. 60/859,284, filed Nov. 14, 2006, with the following modifications: increase the PCR volume from 2 μ l to 4.5 μ l, leading to an increase in the input volume from 4 μ l to 6 μ l. The inlet holes are moved a few millimeters away from the edge of the cartridge to allow room for a 2 mm alignment ledge in the cartridge. A similar alignment ledge is also included on the other edge of the cartridge. (FIGS. 31A, 31B)

Enclosure:

The design of the apparatus enclosure must satisfy requirements: for customer safety during operation; provide access to power and communication interfaces; provide air entry, exit, and filtering; provide one-handed operation to open for installation and removal of materials; incorporate marketable aesthetics.

Cooling:

The cooling for the apparatus will be designed in conjunction with the enclosure and overall system to ensure all assemblies requiring air are within the flow path or receive diverted air.

Of The current concept is for the air inlet to be located on the bottom of the lower front panel. The air will then pass through a cleanable filter before entering the apparatus. Sheet metal components will direct the air to both the disposable racks and the main power supply. The air will then be directed through the card cages, around the readers and will exit through slots provided in the top of the enclosure.

Base Plate:

The XYZ stage and frame are mounted to the base plate in a way where there will be no misalignment between the stage, cartridge and the disposable. The enclosure is mounted to the base plate. Final design of the enclosure determines the bolt hole pattern for mounting. The backplane board mounts to the base plate with standoffs. All other boards mount to the backplane board. The disposable mounts on a rack which will be removable from the brackets mounted to the base plate. The reader brackets bolt to the base plate. Final design of the reader brackets determines the

US 10,632,466 B1

69

bolt hole pattern. The power supply mounts to the base plate. The base plate extends width and lengthwise under the entire instrument.

Example 12: Exemplary High-efficiency Diagnostic Apparatus

A more highly multiplexed embodiment, also enables 24 clinical samples to be automatically processed to purify nucleic acids, mix the purified DNA/RNA with PCR reagents and perform real-time PCR in a microfluidic cartridge. This product has a single PCR reader, with a scanning read-head, capable of reading up to 4 different colors from each of the PCR lane. The cartridge has 24 PCR channels enabling a single cartridge to run all 24 clinical samples. In addition, this product has a cartridge autoloader, whereby the instrument automatically feeds the PCR reader from a pack of cartridges into the instrument and discard used cartridge into a waste tray. Diagrams are shown in FIGS. 73, and 74.

The apparatus has the following sub-systems:

Two sample processing racks, each rack processes up to 12 clinical samples in unitized disposable strips

Magnetic separator-cum-tube heater assembly (24 heating stations)

A four-probe liquid dispensing head

3-axis gantry to move the pipette head

A single PCR amplification-detection station capable of running a 24-lane microfluidic cartridge and a scanner unit to detect up to 4 colors from each PCR lane.

An autoloader unit to feed 24-lane microfluidic cartridge from a box into the PCR detection unit.

Control electronics

Barcode reader

Operation: The user will get a work list for each sample, whether they want to detect certain target analyte (such as CBS, Chlamydia, Gonorrhea, HSV) for each clinical sample. The sample tubes are placed on the rack and for each sample, the user slides in a unitized reagent disposable (analyte specific) into corresponding lane of the rack. The unitized disposable will have all the sample prep reagents, PCR reagents, process tubes as well as disposable pipettes already prepackaged in it. Once all disposables are loaded into the rack, the rack is placed in its location on the instrument. The user then closes the door of the instrument and then starts the sample processing using the GUI (Graphical User Interface).

The instrument checks functionality of all subsystems and then reads the barcode of the sample tubes, the unitized reagent disposables and presence of a 24-lane microfluidic cartridge. Any mismatch with a pre-existing work list is determined and errors are flagged, if necessary. The instrument then goes through a series of liquid processing, heating, magnetic separation to complete the sample preparation steps for the each of the clinical sample, mixes the purified nucleic acid with PCR reagents and dispenses the final mix into a lane of a 24-lane microfluidic cartridge. After the microfluidic cartridge is loaded with the final PCR mix, the cartridge is moved and aligned by an automated motorized pusher in the PCR reader. The optical detection system, then presses the cartridge against a microfluidic PCR heater surface, On-chip valves are actuated to close the reaction mix and then thermo-cycling is started to initiate the PCR reaction. At each cycle of PCR (up to 45 cycles), fluorescence from each PCR lane is detected by the optical detection system (2-colors per PCR lane) and final result is

70

determined based on the threshold cycle (Ct). The used cartridge is then pushed out automatically into a waste cartridge bin.

Microfluidic cartridges are stored in a cartridge pack (maximum 24 cartridges) and the instrument alerts the user to replace the cartridge pack and empty out the waste cartridge bin once all cartridges from the pack are used up. 24-Lane Cartridge

The 24-lane cartridge has two rows of 12 PCR lanes. Various views are shown in FIGS. 75-77. The cartridge has 3 layers, a laminate, a substrate, and a label. The label is shown in two pieces. Each Lane has a liquid inlet port, that interfaces with a disposable pipette; a 4 microliter PCR reaction chamber (1.5 mm wide, 300 microns deep and approximately 10 mm Long), two micro valves on either side of the PCR reactor and outlet vent. Microvalves are normally open and close the channel on actuation. The outlet holes enables extra liquid (~1 μ l) to be contained in the fluidic channel in case more than 6 μ l of fluid is dispensed into the cartridge,

The inlet holes of the cartridge are made conical in shape and have a diameter of 3-6 mm at the top to ensure pipettes can be easily landed by the fluid dispensing head within the conical hole. Once the pipette lands within the cone, the conical shape guides the pipette and mechanically seals to provide error free dispensing or withdrawal of fluid into the cartridge. The bigger the holes, the better it is to align with the pipette, however, we need to maximize the number of inlet ports within the width of the cartridge as well as maintain the pitch between holes compatible with the inter-pipette distance. In this particular design, the inter-pipette distance is 18 mm and the distance between the loading holes in the cartridge is 8 mm. So lanes 1, 4, 7, 11 are pipetted into during one dispensing operation; lanes 2, 5, 8 and 12 in the next, and so on and so forth.

The height of the conical holes is kept lower than the height of the ledges in the cartridge to ensure the cartridges can be stacked on the ledges. The ledges on the two long edges of the cartridge enable stacking of the cartridges with minimal surface contact between two stacked cartridges and also help guide the cartridge into the reader from cartridge pack (cf. FIGS. 28-33).

Cartridge Autoloader

The Cartridge autoloader consists of a place for positively locking a pack of 24 microfluidic cartridges, pre-stacked in a spring-loaded box (e.g., FIG. 33). The box has structural elements on the sides to enable unidirectional positioning and locking of the box in the autoloader (FIG. 33). To load a new box, the user moves a sliding element to the left of the autoloader, places and pushes the box in the slot and releases the sliding lock to retain the box in its right location. Springs loaded at the bottom of the box helps push the box up when it needs to be replaced. The spiral spring present at the bottom of the cartridge pack pushed against the cartridges and is able to continually push the cartridge with a force of from 4 to 20 pounds.

The presence or absence of cartridges is detected by reading the barcode on top of the cartridge, if present.

To start a PCR run, the pipette head dispenses PCR reaction mix into the required number of lanes in the top cartridge in the autoloader (e.g., FIG. 28). The pusher pushes the top cartridge from the autoloader box into the two rails that guide the cartridge into the PCR reader. The cartridge is pushed to the calibrated location under the reader and then the optics block is moved down using a stepper motor to push the cartridge against the microheater surface. The bottom of the optics block (aperture plate) has projections on

the sides to enable the cartridge to be accurately aligned against the apertures. The stepper motor pushes the cartridge to a pre-calibrated position (e.g., FIG. 30) which provides a minimum contact pressure of 1 psi on the heating surface of the microfluidic cartridge.

After the PCR reaction is complete, the stepper motor moves up 5-10 mm away from the cartridge, relieves the contact pressure and enables the cartridge to travel in its guide rails. The pusher is activated and it pushes the cartridge out to the cartridge waste bin (e.g., FIG. 32). After this step, the pusher travels back to its home position. During its back travel, the pusher is able to rise above the top of the cartridge in the cartridge pack because it has an angular degree of freedom (see figure). A torsion spring ensures the pusher comes back to a horizontal position to enable it to push against the next cartridge in queue. The pusher is mechanically attached to a timing belt. The timing belt can be moved in either direction by turning a geared motor. The pusher is mounted to a slider arrangement to constrain it to move in only one axis (see, e.g., FIG. 31).

The cartridge pushing mechanism can also be made to not only push the cartridge from the autoloader box to the detection position, but also be used to move it back to the autoloading position. This will enable unused lanes in the microfluidic cartridge to be used in the next PCR run.

The cartridge autoloading box is also designed so that once all the cartridges are used, the box can be easily recycled or new cartridges added to it. This reduces the cost to the customer and the manufacturer.

Reader

The reader consists of an optical detection unit that can be pressed against a 24-lane microfluidic cartridge to optically interface with the PCR lanes as well as press the cartridge against a microfluidic heater substrate (FIG. 78). The bottom of the optics block has 24 apertures (two rows of 12 apertures) that is similar in dimension of the PCR reactors closest to the cartridge. The aperture plate is made of low fluorescent material, such as anodized black aluminum and during operation, minimized the total background fluorescence while maximizing the collection of fluorescent only from the PCR reactor (FIGS. 79A and 79B). The bottom of the aperture plate has two beveled edges that help align two edges of the cartridges appropriately such that the apertures line up with the PCR reactors. (FIGS. 80, 81).

The optical detection units (total of 8 detection units) are assembled and mounted onto a sliding rail inside the optical box so that the optical units can be scanned over the apertures (FIG. 82). Each unit is able to excite and focus a certain wavelength of light onto the PCR reactor and collect emitted fluorescence of particular wavelength into a photodetector. By using 4 different colors on the top 4 channels and repeating the 4 colors in the bottom channels, the entire scanner can scan up to 4 colors from each of the PCR lanes.

The optics block can be machined out of aluminum and anodized or injection molded using low fluorescence black plastic (FIG. 83). Injection molding can dramatically reduce the cost per unit and also make the assembly of optics easier. The designed units can be stacked back-to-back.

Example 13: Exemplary Electronics for Use with Preparatory and Diagnostic Apparatuses as Described Herein

There are multiple independent software modules running on dedicated hardware: Described herein exemplary specifications for the electronics used in the diagnostic (PCR) system. Additional information related to the PCR System is

described elsewhere herein. In some embodiments, the PCR system includes eighteen printed circuit boards (PCBs) of nine different types. Referring to FIG. 86, the system can contain three multiplex (MUX) boards 100a-c, two of which (micro-heater MUX boards 100a-b), can each be used to run a micro-heater board 110a-b and the third (lysis heater MUX board 100c) can run one or more lysis heater boards 116 and 117. Each of the three MUX boards 100a-c can be controlled by a PC processor board via an Ethernet port. The two micro-heater boards 100a-b, each controlled by one of the MUX boards 100a-b, heat micro-zones on the microfluidic cartridge. In some embodiments, the system includes the two lysis heater boards 116 and 117, controlled by the lysis heater MUX board 100c, that heat lysis tubes in each of the two 12 sample racks.

Still referring to the PCBs included in the PCR system, the system can include two 12-channel optical detection boards 130a-b that can each detect optical fluorescence emitted by microfluidic cartridge chemistry. The optical detection boards can be controlled by one or more of the MUX boards 100a-c, using SPI, over a RS-422 interface. The system can include three motor control boards 140a-c, where one board (e.g., motor control board 140c) can control two magnetic separation motors (not shown), and the remaining two motor control boards (e.g. motor control boards 140a-b) can each run one reader tray motor (not shown) and one reader pressure motor (not shown). The motor control board running the magnetic separation motors (e.g., motor control board 140c) can be controlled via RS-485 interface from the lysis heater MUX board 100c and the two motor control boards 140a-b, each running one reader tray motor and one reader pressure motor, can be controlled via RS-485 interface by the micro-heater MUX boards 100a-b. The system can also include one PC processor board 150, which directs the overall sequencing of the system and can be controlled via external Ethernet and USB interfaces, and one PC processor base board 160, which provides internal interfaces for the PC processor board ISO to the remainder of the system and external interfaces. The system can include one main backplane 180 that interconnects all system boards, one motor control backplane 190 that interconnects the motor control boards 140a-c to the main backplane 180 and gantry (not shown), and two door sensor boards (not shown). One door sensor board provides an interconnect between the front door solenoid locks and the PC processor base board 160 and the other door sensor board provides an interconnect between the position sensors and the PC processor base board 160.

In some embodiments, the PCR system can include the off-the-shelf PC processor board 150. The PC processor board 150 can be an ETX form factor board that includes one 10/100 BASE-T Ethernet port, four USB ports, one analog VGA display port, two UART ports, one real-time clock, one parallel port, one PS2 keyboard port, one PS2 mouse port, stereo audio output, one IDE interface, and one 12C interlace.

Referring to FIG. 87, the system can also include the PC processor base board 160 that includes a five port 10/100 BASE-T Ethernet bridge 161 for internal communication, one of which can be connected to the 10/100 BASE-T Ethernet port of the PC Processor board 130 another of which can be for diagnostic use (with a connector inside system cover), and three of which can communicate with the three MUX boards 100a-c (one port for each MUX board 100a-c) through the backplane 180. The PC processor base board 160 can also include one USB to 10/100 BASE-T Ethernet port 162 for external Ethernet connections, one four

US 10,632,466 B1

73

port USB hub **163** for external connections, one external VGA connector **164**, one internal PS2 Mouse connector **165** (with a connector inside the system cover), and one internal PS2 Keyboard connector **166** (with a connector inside the system cover). The PC processor base board **160** can also include one internal stereo audio output **167** to on board speakers **168**, one internal CompactFlash connector **169** from an IDE port (with a connector inside the system cover), and one internal RS-232 interface **170** from a UART port (with a connector inside the system cover). Additional components included in the PC processor base board can include one internal RS-485 interface **171** from a UART port (with a connector inside the system cover), one internal temperature sensor **172** connected to the 12C interface, a battery for the real-time clock, and one parallel port **173**. The parallel port **173**, with connectors inside the system cover, can be internally connected as follows; one bit can be used to drive a high current low side switch for the two door solenoids, one bit can be used to generate a processor interrupt when either door sensor indicates that a door is opened, three bits can be used to program the EEPROM for configuring the Ethernet bridge **161**, and two bits can be connected to the Ethernet bridge management interface (not shown). The remaining bits can remain unassigned, with optional pull-up and pull-down resistors, and be brought out to a 10 pin Phoenix contact header.

Referring now to FIG. **88**, in some embodiments, the system can include the three MUX boards **100a-c**. While FIG. **88** depicts exemplary MUX board **100a**, each of the three MUX boards **100a-c** can include one or more of the features described below. The MUX board **100a** can include 96 pulse width modulated (PWM) controlled heating channels with heaters (about 33 ohm to about 150 ohm) heaters, that can support 20 or 24 volt (voltage externally provided) drives with a maximum current of about 800 mA. Each PWMs can be 12-bit with programmable start and stop points, can have 1 microsecond resolution, and can have a maximum duty cycle of about 75%. Each PWM period is programmable and is preferably set to 4 ms. The MUX boards can include a 4-wire RTD/heater connection with precision 1 mA sense current that can accommodate about 50 ohm to about 2500 ohm resistive temperature devices and have a measurement accuracy of ± 0.5 ohms. The thermal measurement sample period of the MUX boards is 32 ms including 8X PWM periods where 12 16-bit ADCs **101a** sample 8 successive channels each. The MUX address can be tagged to the ADC data.

Still referring to the MUX board **100a** depicted in FIG. **88**, an RS-422 optics board interface **102a** that interconnects over the backplane **180** and transfers data over a 4 wire SPI interface using local handshake signals and interrupts can be included on the MUX board **100a**. The MUX board **100a** can also include a 10/100 BASE-T Ethernet interface **103a** that interconnects to the system over the backplane **180** and an RS-485 interface **104a** that interconnects to the motor controller **140a** over the backplane **180**.

Referring now to FIG. **89**, in some embodiments, the system can include the optical detection boards **130a-b**. While FIG. **89** depicts exemplary optical detection board **130a**, each of the optical detection boards **130a-b** can include one or more of the features described below. The optical detection board **130a** can include a 12-channel optics board design modified to use art RS-422 interface **131a**. The optical detection board **130a** can include 12-3 Watt, blue LEDs **132a** driven with about 6 V at about a 625 mA maximum. An exemplary LTD used in the detection board **130a** is the Luxeon K2 emitter producing blue light at a

74

wavelength of about 470 nm using about 27 mW @700 mA. The optical detection board **130a** can also include 12-3 Watt, amber LEDs **133a** driven with about 6 V at about a 625 mA maximum. An exemplary LED used in the detection board **130a** is the Luxeon K2 emitter producing amber light at a wavelength of about 590 nm using about 60 mW @700 mA. The detection board **130a** can include 24 lensed silicon photodiode detectors **134a**, an example of which is the Hamamatsu S2386-18L. These photodiode detectors **134a** are designed in a common TO-18 package. The detection board **130a** can also include an MSP430 processor **135a** with two PWM channels, one for the blue channel and one for the amber channel. The board **130a** can include individual LED enables **136a** and **137a** for each of the 12 color pairs set over the local SPI bus.

The PCR system can include a lysis heater board that provides and monitors heating to the lysis tubes. The heater board can include 12-70 Watt TO-247 power resistors (provide heat to the lysis tubes) designed to be fed 24V from one or more of the MUX boards **100a-c** (e.g., MUX board **100c**) and 12-2000 ohm Resistive Temperature Devices (RTD) to monitor the temperature of the lysis tubes. Optional resistors can be included to modify the full scale range of the RTDs. Included on the lysis heater board is a serial EEPROM that may hold a board serial number and can be used to identify the board type and revision level to software.

Referring now to FIG. **90**, in some embodiments, the system can include the micro-heater boards **11a-b**. While FIG. **90** depicts exemplary micro-heater board **110a**, each of the micro-heater boards **11a-b** can include one or more of the features described below. In some embodiments, the system can include the micro-heater board **110a** that includes a serial EEPROM and two optical interrupters. The serial EEPROM may hold a board serial number, can hold RTD calibration data, and can be used to identify the board type and revision level to software. The optical interrupters can be used to sense the reader tray position for the motor control board **140a** and sends the information to the Blue Cobra (motor controllers), which processes the information on the positions of the reader trays and accordingly controls the power to the emitters supplied by the motor control board **140a**. The micro-heater board **110a** can provide connections to the 96 channel micro-heater plate and control the 96 multiplexed heater/RTD devices to control cartridge feature temperature. The heater/RTD devices can be between about 50 ohms to about 500 ohms. The micro-heater board **110a** can bridge the RS-422 interface from, for example, the MUX board **100a** to the optical detection board **130a**. The connection from the micro-heater board **110a** to the MUX board **100a** is over the backplane **189**, while the connection to the optics board **130a** is over a 40 pin FFC cable.

Referring now to FIG. **91**, in some embodiments, the system can include the motor control boards **140a-c**. While FIG. **91** depicts exemplary motor control board **140a**, each of the motor control boards **140a-c** can include one or more of the features described below. In some embodiments, the system can include the motor control board **140a** that can control two micro-stepping motors **141a** and can be connected to the backplane **180** via a RS-485 interface. The output to the motors can be up to 24 V supplied externally through the backplane **180**. The output current can be jumper selectable. Exemplary output currents that can be selected via jumper settings can include about 700 mA, about 1.0 A, or 2.3 A. The motor control board **140a** includes open collector TTL interrupt output to the MUX board **100a**

US 10,632,466 B1

75

and flag inputs. The flag inputs can provide 1.5 V power output to the sensors and can be switched on and off by software.

Limit switches are placed on the extreme locations of each axis, e.g., x-minimum and x-maximum, that turns off the power to the motor driving that axis in case of a malfunction happens and the pipette head moves out of the designed working distance. Optional pull-up and pull-down are used with the output of the optical interrupters.)

In some embodiments, the system can include one or more interconnection boards, such as the main backplane **180**. The main backplane **180** can interconnect other PCBs, such as the MUX boards **100a-c**, PC processor base board **160**, and heater Interconnect boards. The main backplane **180** can cable to the motor control backplane **190** and to two lysis heater boards. The main backplane **180** can distribute power and signaling, implement 10/100 BASE-T Ethernet and RS-485 over the backplane **180**, and supplies voltages from an external connector. Exemplary voltages supplied include +3.3 V, +5.0 V, +12.0 V, -12.0 V, +20.0 V, and +24.0 V.

The system can include the motor control backplane **190** that can distribute power and signaling for all of the motor control boards **140a-c**. The motor control backplane **190** can supply +5.0 V and 24.0 V from an external connector. The motor control backplane **190** can include 1 slot for the RS-485 signaling from each of the two MUX boards **100a-b** (total of 2 slots). 6 slots for the RS-485 signaling from the lysis heater controlling MUX board **100c**, and one connector that provides RS-485 signaling and power to the gantry. The motor control backplane **190** can provide pull-up and pull-down resistors to handle floating buses.

In some embodiments, the system can include a heater interconnect board and a door sensor board. The heater interconnect board can connect the micro-heater boards **110a-b** to the main backplane **180** using a physical interconnect only (e.g., no active circuits). The door sensor board can provide a cable interlace and mixing logic from the optical interrupters, which sense the door is open, and provide a mounting and cabling interlace to the door lock solenoid.

Example 14: Exemplary Software for use With Preparatory and Diagnostic Apparatuses as Described Herein.

There are multiple independent software modules running on dedicated hardware;

Reader(2);
Sample-Prep (1);
User Interface (1);
Detector(2);
Motor control (8)

Inter-module communication among is via an internal Ethernet bus, communication with the user interface is via a high speed SPI bus and communication with motor control via a RS485 serial bus.

The Reader and Sample-Prep software run on identical hardware and are as such identical incorporating the following functions;

Script Engine (a parametrized form of a protocol)
Protocol Engine
Temperature Control (Microfluidics, lysis, release)
Motor control (via external motor control modules).
Salient features of the motor control software are:
Command/reply in ASCII and addressing capability to allow daisy chaining of communication link;

76

Detection (via external detector modules). Detector module controls the LED illumination and photo detector digitization.

The user interface is implemented as a program running under Linux operating system on an embedded x86 compatible PC. The following functions are addressed;

Graphical User Interface
Test result storage and retrieval Network connectivity via Ethernet (to lab information systems)
USB interface
Printer
Scanner (Internal and external)
Keyboard
Mouse
Door lock and sense

Example 15: Exemplary Chemistry and Processes of Use

Chemistry Overview

The chemistry process centers around the detection and identification of organisms in a clinical specimen, by virtue of detecting nucleic acids from the organism in question. This involves isolation of nucleic acids from target organisms that are contained in a clinical specimen, followed by a process that will detect the presence of specific nucleic acid sequences. In addition to target detection, an internal positive control nucleic acid will be added to the collection buffer, and will be taken through the entire extraction and detection process along with target nucleic acids. This control will monitor the effectiveness of the entire process and will minimize the risk of having false negative results. Nucleic Acid Extraction and Purification:

Nucleic acid extraction procedures begin with the addition of a clinical specimen to a prepared specimen collection solution. This can be done either at a specimen collection site, or at the testing site. Two collection solution formats will be available: one for body fluids, and one for swab specimens. Collection solutions used at collection sites will serve as specimen transport solutions, and therefore, this solution must maintain specimen and analyte integrity.

The extraction and purification procedure, which is entirely automated, proceeds as follows:

Target organisms are lysed by heating the detergent-containing collection solution.

Magnetic beads, added to the specimen/collection solution mix, non-specifically bind all DNA that is released into the solution.

Magnetic beads are isolated and are washed to eliminate contaminants

DNA is released from the beads using high pH and heat. DNA containing solution is removed and neutralized with a buffer

Nucleic Acid Amplification

Nucleic acids that have been captured by magnetic beads, washed, released in high pH, and neutralized with buffer, are added to a mixture of buffers, salts, and enzymes that have been lyophilized in a tube. The mixture is rapidly rehydrated, and then a portion of the solution is loaded onto a microfluidic cartridge. The cartridge is then loaded into the amplification instrument module, which consists of a heating unit capable of thermal cycling, and an optical detection system. Detection of target nucleic acids proceeds as follows:

US 10,632,466 B1

77

The liquid in sealed in a reaction chamber.

Rapid thermal cycling is used to potentiate the Polymerase Chain Reaction (PCR), which is used to amplify specific target DNA.

Amplified DNA fluoresces, and can be detected by optical sensors.

A fluorescent probe "fair" is incorporated into each amplified piece of DNA

At a specific temperature, the probe adopts a conformation that produces fluorescence (this is termed a "scorpion" reaction, see FIG. 84).

Fluorescence is detected and monitored throughout the reaction.

Extraction and Amplification/Detection Process:

Extensive bench-scale testing has been performed to optimize the nucleic acid extraction chemistry, including the collection buffer, the wash buffer formulation, the release solution formulation, and the PCR reagent mixes. The fully automated method of extraction, followed by 12-up PCR, was able to provide very high sensitivity consistently at 150 copies/sample,

Examples: Chlamydia in Urine (50/50); Gonorrhea in Urine; GBS in Plasma.

Various detection chemistries such as Taqman, Scorpion, SYBRg Green work reliably in the microfluidic cartridge. Reagent Manufacturing

Feasibility studies were conducted in order to determine whether PCR reagents could be lyophilized in PCR tubes besides the use of 2 µl lyophilized pellets. The studies have indicated that sensitivity of reactions performed using tube-lyophilized reagents is equivalent to that of wet reagents or 2 µl pellet reagents, so feasibility has been proven. Stability studies for this format indicate similar stability data. We have seen 2 microliter lyophilized PCR pellets to be stable to up to 2 years at room temperature, once sealed in nitrogen atmosphere.

Manufacturing Overview: Manufacturing the components of the system can be accomplished at HandyLab, Inc., Ann Arbor, Mich. The manufacturing task has been split into five areas that consist of: chemistry manufacture, disposable strip, collection kit, cartridge and analyzer.

Chemistry Manufacturing: There are currently seven individual, blended chemistry components identified for potential use with the system described herein. Mixing, blending and processing reagents/chemicals can be performed at HandyLab, Inc., with existing equipment already in place. Additional tooling and fixtures will be necessary as the product matures and we ramp to high volume production, but initial costs will be minimal.

Collection buffer, wash, release & neutralization liquids are simple recipes with very low risk, and can be made in large batches to keep labor costs of mixing/blending at or below targeted projections. They will be mixed and placed into intermediate containers for stock, and then issued to Disposable Ship Manufacturing for dispensing. Mature SOP's are in place from prior project activity.

Affinity Beads (AB) have good potential to be stored and used as a liquid in the strip, but design contingencies for using a lyophilized pellet are in place as a back up. It is critical to keep the beads suspended in solution during dispense. Dispense equipment (e.g., manufactured by Innovadyne) that provides agitation for continuous suspension during dispense has been identified for purchase once stability has been proven for liquid AB storage in the strip. The process to manufacture and magnetize the Affinity Beads

78

spans a 9 hour cycle time to produce a batch of 2,000 aliquots, but that same time period can be used for scaled up recipe batches once we ramp into high volume production. This item has the highest labor content of all chemistry manufacture that is currently required for the apparatus.

PCR reagents/enzymes will be freeze-dried in our existing lyophilizing chamber (Virus Genesis) but will not require spherical pellet formation. Instead, the mixture is being dispensed into, and then lyophilized, inside the end-use tube. First the chemistries are mixed per established SOPs, and then the following steps are performed to accomplish lyophilization. Individual tubes are placed into a rack/fixture, and the solution is dispensed into each, using existing equipment (EFD Ultra Dispense Station.) The filled rack will be placed inside a stainless steel airtight box (modified to accept stoppers in the lid,) and then placed into the lyophilization chamber and the drying cycle commences unattended. During lyophilization, the stoppers are in a raised position allowing air/nitrogen to circulate into, and moisture to exit the stainless box holding racks of vials. At the end of the cycle, the shelves of our lyophilization chamber lower to seat the stoppers into the lid, forming a seal while still inside the closed chamber, in a moisture free nitrogen atmosphere. The steel boxes are then removed from the chamber, and each rack inside shall be processed in a single operation to seal all vials in that rack. Immediately after sealing, the vials will be die cut from the foil in one operation, allowing individual vials to be forwarded to the Disposable Manufacturing area for placement into a strip. Internal Control will either be added to an existing solution, or will be dispensed into its own cavity in the manner of the collection buffer, wash, neutralization, and release solutions, if lyophilization is required, it will be accomplished in the same manner as the PCR chemistry, and later snapped into the strip. Shelf life stability studies are underway.

Collection Kit Manufacturing

The collection kit will be processed manually in house for initial quantities. Initial quantities will not require capital expenditures as we have all equipment necessary to enable us to meet projections through 2008. We will be using our existing (EFD 754-SS Aseptic Valve & Valvemate 7000 Digital Controller,) to fill the collection vial. The vials have a twist-on top that will be torqued, and the vial will have a proprietary ID barcode on each vial. 24 vials will be placed into a reclosable plastic bag and placed into a carton for shipping.

Place vials into rack.

Dispense solution into vials.

Install and torque caps.

Label vials.

Bag vials and label bag.

Place vial bag and instructions/insert into carton, close and label.

55 Cartridge Manufacturing:

Existing semi-automatic equipment for laminating & waxing (Think & Tinker DF-4200, & Asymick Axiom Heated Jet Platform, respectively,) will be utilized to meet all cartridge manufacture requirements. The footprint of the 12-up disposable is the same as the RTa10 cartridge, so additional fixtures are not necessary.

Laminate micro substrate & trim excess.

Fill valves with hot wax & inspect.

Apply label & barcode.

Band 24 pieces together.

Bag & seal banded cartridges, label bag.

Place bag & insert(s) into carton, seal and label.

US 10,632,466 B1

79

This portion of the product is relatively simple, although there is a difference between the automated (as used herein) and the stand-alone 12-up cartridge. Venting will not be required on the cartridge, which eliminates the most time consuming process for cartridge manufacture, along with the highest risk and highest cost for fully integrated automation. Over 1,000 pieces of the 12-up with venting have been successfully produced.

Example 16: Exemplary Chemistry Processes

Sample Pre-processing

For Urine Sample: Take 0.5 ml of urine and mix it with 0.5 ml of HandyLab collection buffer. Filter the sample through HandyLab Inc.'s pre-filter (contains two membranes of 10 micron and 3 micron pore size). Place the sample tube in the position specified for the external sample tube in the 12-up rack.

For Plasma Sample: Take 0.5 ml of plasma and mix it with 0.5 ml of HandyLab collection buffer. Place the sample tube in the position specified for the external sample tube in the 12-up rack.

For GBS swab samples: Take the swab sample and dip it in 1 ml of HandyLab collection buffer. Place the sample tube in the position specified for the external sample tube in the 12-up rack.

The HandyLab sample collection buffer contains 50 mM Tris pH 7.1% Triton X-100, 20 mM Citrate, 20 mM Borate, 100 mM EDTA, plus 1000 copies of positive control DNA.

Limiting the Instrument and Starting Sample Processing

1. Load PCR lube containing PCR master mix in one of the specified snap-in location of the unitized disposable.
2. Load PCR tube containing PCR probes and primers for the target analyte under consideration in the specified location of the unitized disposable.
3. In case of two analyte test, load PCR tube containing probes and primers for second analyte in the specified location of the unitized disposable.
4. Load the unitized disposable in the 12-up rack in the same lane as the sample tube under consideration.
5. Prepare and load unitized reagent strips for other samples in consideration.
6. Load the 12-up rack in one of the locations in the instrument.
7. Load 12-up cartridge in the cartridge tray loading position.
8. Start operation.

Liquid processing steps

1. Using Pipette tip#1, the robot transfers the clinical sample from the external sample tube to the lysis tube of the unitized disposable strip.
2. Using the same pipette tip, the robot takes about 100 µl of sample, mixes the lyophilized enzyme and affinity beads, transfers the reagents to the lysis tube. Mixing is performed in the lysis tube by 5 suck and dispense operations.
3. The robot places pipette tip#1 at its designated location in the unitized disposable strip.
4. Heat the lysis tube to 60° C. and maintain it for 10 minutes.
5. After 5 minute of lysis, the robot picks up pipette tip#1 and mixes the contents by 3 suck and dispense operations.
6. The robot places pipette tip#1 at its designated location in the unitized disposable strip.

80

7. After 10 minutes of lysis, a magnet is moved up the side of the lysis tube to a middle height of the sample and held at that position for a minute to capture all the magnetic beads against the wall the tube.
8. The magnet is brought down slowly to slide the captured beads close to the bottom (but not the bottom) of the tube.
9. Using pipette tip#2, aspirate all the liquid and dump it into the waste tube.
10. Aspirate a second time to remove as much liquid as possible from the lysis tube.
11. Using the same pipette tip#2, withdraw 100 µl of wash buffer and dispense it in the lysis tube. During this dispense, the magnet is moved downwards, away from the lysis tube.
12. Perform 15 mix steps to thoroughly mix the magnetic beads with the wash buffer.
13. Wait for 30 seconds.
14. Move magnet up to capture the beads to the side and hold for 15 seconds/
15. Using pipette tip#2, aspirate wash buffer twice to remove as much liquid as possible and dump it back in the wash tube.
16. Move magnet down away from the lysis tube.
17. Place pipette tip#2 in its specified location of the unitized disposable strip.
18. Pick up a new pipette tip (tip #3) and withdraw 8-10 µl of release buffer and dispense it over the beads in the lysis tube.
19. Wait for 1 minute and then perform 45 mixes.
20. Heat the release solution to 85° C. and maintain temperature for 5 minutes.
21. Place pipette tip#3 in its specified location of the unitized disposable strip.
22. Bring magnet up the tube, capture all the beads against the tube wall and move it up and away from the bottom of the tube.
23. Pick up a new pipette tip (tip#4) and withdraw all the release buffer from the lysis tube and then withdraw 3-10 µl of neutralization buffer, mix it in the pipette tip and dispense it in the PCR tube. (In case of two analyte detections, dispense half of the neutralized DNA solution into first PCR tube and the rest of the solution in the second PCR tube.
24. Using pipette tip#4, mix the neutralized DNA with the lyophilized reagents by 4-5 suck and dispense operations and withdraw the entire solution in the pipette tip.
25. Using pipette tip#4, load 6 µl of the final PCR solution in a lane of the 12-up cartridge.

The usage of pipette heads during various processes is shown schematically in FIGS. 85A-C.

Heal-Time PCR

After all the appropriate PCR lanes of the PCR cartridge is loaded with final PCR solution, the tray containing the cartridge moves it in the PCR Analyzer. The Cartridge is pressed by the Optical detection read-head against the PCR heater. Heaters activate valves to close either ends of the PCR reactor and real-time thermocycling starts. After completing appropriate PCR cycles (~45 cycles), the analyzer make a call whether the sample has the target DNA based on the output fluorescence data.

Pipette Detection

The pipette head has 4 infrared sensors for detecting the presence of pipettes. This is essential to ensure the computer positively knows that a pipette is present or missing. Since pipettes are picked up using mechanical forcing against the

US 10,632,466 B1

81

pipette and also dispensed using mechanical motion of a stripper plate, pipette sensing helps preventing errors that otherwise may happen.

Force Sensing of the Pipette Head

The multi-pipette head is assembled in such a way and a force sensor interfaced with it so that any time the pipette head seats against the disposable pipette(s) or the picked pipettes are forced through the laminate in the reagent disposable or the pipette is forced against the bottom of the tubes in the reagent disposable, an upward force acts on the pipette head through the pipette holding nozzle or the pipettes itself. The entire head is pivoted, as shown in Figure and any force acting on the head causes a set-screw on the upper part of the head to press against a force sensor. This force sensor is calibrated for vertical displacement of the head against a non-moving surface. Using this calibration, it can be determined when to stop moving the head in the z-direction to detect whether pipettes are properly seated or if pipettes hit tube bottoms.

Alignment of Pipette Tips While Loading PCR Reagents into the Microfluidic Cartridge

The pipettes used in the apparatus can have volumes as small as 10 μ l to as large as 1 ml. Larger volume pipettes can be as long as 95 mm (p1000 pipette). When 4 long pipette tips are sprung from the head, even a 1° misalignment during seating can cause the tip to be off-center by 1.7 mm. As it is impossible to have perfect alignment of the tip both at the top where it is interfaced with the tip holder and the bottom, it becomes necessary to mechanically constrain all the tips at another location closet to the bottom. We have used the stripper plate, having a defined hole structure to use it to align all the tips. The stripper plate hole clears all the 4 pipette tips when they are picked up. After the tips are properly seated, the stripper plate is moved in the x-axis using a motor to move all the pipettes against tins notch provided in the stripper plate (see FIG. 46b). Now all the pipettes land on the cartridge inlet holes with ease.

Sample Preparation Extensions

The current technology describes details of processing clinical samples to extract polynucleotides (DNA/RNA). The same product platform can be extended to process samples to extract proteins and other macromolecules by changing the affinity molecules present in the magnetic beads. The amplification-detection platform can also be used to perform other enzymatic reactions, such as immunoPCR, Reverse-transcriptase PCR, TMA, SDA, NASBA, LAMP, LCR, sequencing reactions etc. The sample preparation can also be used to prepare samples for highly multiplexed microarray detections as well.

Example 16: Exemplary Material for RNA-affinity Matrix

An exemplary polynucleotide capture material preferentially retains polynucleotides such as RNA on its surface when placed in contact with a liquid medium that contains polynucleotides mixed with other species such as proteins and peptides that might inhibit subsequent detection or amplification of the polynucleotides.

The exemplary polynucleotide capture material is: Polyamidoamine (PAMAM) Generation 0, available from the Sigma-Aldrich Chemical Company ("Sigma-Aldrich"), product number 412368, PAMAM is a dendrimer whose molecules contain a mixture of primary and tertiary amine groups. PAMAM (Generation 0) has the structure shown herein.

82

The PAMAM, during use, is immobilized on a solid support such as carboxylated beads, or magnetic beads. The polynucleotide, capture material comprises polycationic molecules during an operation of polynucleotide capture. Affinity between the material and polynucleotides is high because polynucleotides such as DNA and UNA typically comprise polyanions in solution.

After polynucleotide molecules are captured on a surface of the material, and remaining inhibitors and other compounds in solution have been flushed away with an alkaline buffer solution, such as aqueous 0.1 mM Tris (pH 8.0), the polynucleotides may themselves be released from the surface of the material by, for example, washing the material with a second, more alkaline, buffer, such as Tris having a pH of 9.0.

Exemplary protocols for using PAMAM in nucleic acid testing are found in U.S. patent application Ser. No. 12/172,214 filed Jul. 11, 2008, incorporated herein by reference.

Example 17: Exemplary material for DNA-affinity Matrix

The exemplary polynucleotide capture material is: Polyethyleneimine (PEI), available from the Sigma-Aldrich Chemical Company ("Sigma-Aldrich"), product number 408719.

Exemplary protocols for using PEI in nucleic acid testing are found in U.S. patent application Ser. No. 12/172,208 filed Jul. 11, 2008, incorporated herein by reference.

Example 18: Exemplary Apparatus

Described herein are exemplary specifications for the mechanical design of the PCR system. In some embodiments, the system can be about 28.5 inches deep, or less, and about 43 inches wide, or less, and weight about 250 pounds or less. The system can be designed with a useful life of about 5 years (e.g., assuming 16,000 tests per year) and can be designed such that the sound level for this instrument (during operation) does not exceed 50 dB as measured 12 inches from the instrument in all ordinate directions. In some embodiments, the exterior of the system can be white with texture.

Referring to the overall system, in some embodiments, critical components of the system can remain orthogonal or parallel (as appropriate) to within 0.04 degrees. Exemplary critical components can include motion rails, pipettes, nozzles (e.g., axially as individual nozzles, linearly as an array of four nozzle centroids, or the like), lysis heaters, major edges of the installed cartridge holder in the reader drawer, the front face of the separation magnets, and the like. In the following descriptions, the X-axis (or X direction) refers to the axis extending from left to right when facing the front of the system, the Y-axis (or Y direction) refers to the axis extending from back to front when facing the front of the system, and the Z-axis (or Z direction) refers to the axis extending up from the bottom when facing the front of the system. As viewed from the top of the instrument, the centroid of the leftmost pipette nozzle on the Z-payload (as viewed from the front of the instrument) can be capable of unobstructed travel in the X direction from a point 80 mm from the outermost left baseplate edge to a point 608 mm from the outermost left baseplate edge and can be capable of unobstructed travel in the Y direction from a point 60 mm from the outermost front baseplate edge to a point 410 mm from the outermost front baseplate edge.

US 10,632,466 B1

83

Still referring to the system, as viewed from the front of the instrument, the bottom-most face of the pipette nozzles on the Z-payload can be capable of unobstructed travel in the Y direction from a point 156 mm above the top surface of the baseplate to a point 256 mm above the top surface of the baseplate. The 1 ml pipette tips can be capable of penetrating the foil covers included on disposable reagent strips. This penetration may not create contamination, affect the associated chemistries, or damage the pipette tips. Motions can be executed in such a manner as to eliminate mechanical hysteresis, as needed. Gantry motions can be optimized to prevent cross lane contamination and carryover. The rack can align the reagent strips to a tolerance of ± 0.010 inches in the X and Y directions.

Referring now to the gantry, in some embodiments, the gantry can consist of a stepper-motor actuated, belt/screw-driven cartesian robotic system. The gantry can be free to move, with or without attachments, above the modules that are forward of the rear facade and below the bottom-most horizontal face on the Z head, so long as the Z-payload is fully retracted. The gantry can be capable of travel speeds up to about 500 mm/sec in the X and Y directions and up to about 100 mm/sec in the Z direction. The accuracy and precision of the axis motions (e.g., with respect to the X, Y, and Z home sensors) can be 25 mm or better for each axis, and can be retained throughout the maintenance period. The axis drive belts may not leave residue in areas where PCR and samples are processed. The gantry can contain provisions for routing its own and all Z-payload wire harnesses back to the instrument. Belt tension, on the X and Y axes can be set at 41.5 \pm 3.5 pounds.

Referring now to the Z-payload, the fluid head can have 4 pipette attachment nozzles located on 24 mm centers. Exemplary pipette tips that the pipette nozzles can capture without leakage include Biorobotix tips PN23500048 (50 μ L), PN23500049 (1.75 μ L), and PN23500046 (1 ml). The Z payload can incorporate a stepper actuated stripper plate capable of removing pipette tips (e.g., the pipette tips described above). The system can include a pump and manifold system that includes software controlled aspiration, dispensing, and venting of individual fluid volumes within each of the four individual tips and simultaneous dispensing and venting on all tips. The pump and manifold system can have an accuracy and precision of about ± 2 μ L per tip for volumes that are less than 20 μ L and about $\pm 10\%$ for volumes greater than or equal to 20 μ L (e.g., when aspirating or dispensing in individual tips). The total pump stroke volume can be greater than about 8 μ L and less than about 1250 μ L. The minimum aspirate and dispense speed can be about 10 μ L/sec to about 300 μ L/sec. The centroid of the bottom-most face of each pipette tip can be axially aligned with the nozzle centroid of the pipette nozzles within 0.2 mm. The bottom-most pipette tip faces can be co-planar within 0.2 mm. The Z-payload can incorporate a Z axis force sensor capable of feedback to software for applied forces of between about 0 and 4 lbs. The Z-payload can incorporate a downward facing barcode reader capable of reading the system barcodes as described elsewhere herein.

Referring now to racks included in the system, disposable reagent strips (e.g., oriented orthogonally to the front of the instrument) can be contained in 2, 12-lane racks. The 12 reagent strips in a given rack can register and lock into the rack upon insertion by a user. The rack can contain an area for 12 sample lysis tubes (e.g., PN 23500043) and hold the tube bottoms co-planar, allowing the user to orient the bar code to face the rear of the instrument. Certain features,

84

including those listed above, can allow the racks to be inserted and oriented in the instrument by a minimally trained user. Proper rack placement can be confirmed by feedback to the software. In some embodiments, the racks can be black and color fast (e.g., the color may not appreciably degrade with use or washing with a 10% bleach solution) and the rack material can be dimensionally stable within 0.1 mm over the operating temperature range of the system. The rack can be designed with provisions to allow the rack can be carried to and from the instrument and to minimize or eliminate the likelihood that the tubes held by the rack will spill when placed on a flat surface.

Referring now to the reader and PCR heater included in the system, the reader can allow for cartridge insertion and removal by, for example, a minimally trained user. The cartridge can remain seated in the reader during system operation. In some embodiments, the cartridge barcode may not be read properly by the barcode scanner if the cartridge is inserted incorrectly (e.g., upside down or backwards), thus the system can instruct a user to correctly reinsert the cartridge into the reader tray when the cartridge is inserted incorrectly. The reader drawer can repeatedly locate the cartridge, for loading by the pipette tips, within 0.5 mm. The reader can deliver the cartridge from the loading position into a react and detect position by means of an automated drawer mechanism under software control. The PCR lanes of the cartridge can be aligned, with both the optical system and heater, by the reader tray and drawer mechanism. The cartridge cart contact the heaters evenly with about a 1 psi, or greatest, average pressure to the areas of the PCR channels and the wax valves. Heater wire bonds can be protected from damage so as not to interfere with system motion. Registration from heater to cartridge and from cartridge to optical path centers can be within ± 0.010 inches. The reader can mechanically cycle a minimum of about 80,000 motions without failure.

Referring now to the one or more lysis heaters included in the system, the heaters for each of the 24 lysis stations can be individually software controlled. The lysis ramp times (e.g., the time that it takes for the water in a lysis tube to rise from a temperature of approximately 2.5° C. to a given temperature) can be less than 120 seconds for a rise to 50° C. and less than 300 seconds for a rise to 75° C. The lysis temperature (e.g., as measured in the water contained in a lysis tube) can be maintained, by the lysis heaters, within $\pm 3^\circ$ C. of the desired temperature. The accessible lysis temperature range can be from about 40° C. to about 82° C. Each of the lysis heaters may draw about 16 Watts or more of power when in operation. The lysis heater can be designed to maximize the thermal transfer to the lysis tube and also accommodate the tolerances of the parts. The lysis heaters can permit the lysis tubes to be in direct contact with the magnets (described in more detail herein). The lysis heaters may be adjustable in the horizontal plane during assembly and may not interfere with the installed covers of the system.

Referring now to magnets included in the system, the lysis and magnet related mechanisms can fit beneath the rack and may not interfere with rack insertion or registration. The magnets may be high-flux magnets (e.g., have about a 1,000 gauss, or greater, flux as measured within a given lysis tube) and be able to move a distance sufficient to achieve magnetic bead separation in one or more of the lysis tubes filled to a volume of 900 μ L. The magnets can be software-controllable at movement rates from about 1 mm/sec to about 25 mm/sec. The wiring, included as part of the heater and controller assemblies, can be contained and protected from potential spills (e.g., spills of the lysis tubes). The magnets

US 10,632,466 B1

85

can be located about 1.25 inches or greater from the bottom of the lysis tube when not in use and can be retained in such a manner as to maximize contact with the lysis tube while also preventing jamming.

In some embodiments, the system enclosure includes a semi-transparent lid (e.g., with opaque fixtures and/or hardware) in the front of the instrument to allow users to view instrument functions. The lid can include a company and/or product logo and a graspable handle (e.g., enabling the user to raise the lid). When closed, the lid can have an opening force no greater than 15 pounds (e.g., when measured tangential to door rotation at the center of the bottom edge of the handle) and can lock in the open (e.g., "up") position such that no more than about 5 lbs. of force (e.g., applied at the handle and tangential to door rotation) is required to overcome the handle lock and return the lid to the closed position. The lid can include two safety lid locks that are normally locked when power is not applied and can allow the system to monitor the state (e.g., open or closed) of the lid. The lid can be designed such the lid does not fall when between the open and closed positions. The enclosure can include a power switch located on the right side of the instrument. A power cord can protrude from the enclosure in such a way that positioning the instrument does not damage the cords or cause accidental disconnection. The enclosure can prevent the user from coming in contact with, for example, moving parts, high magnetic fields, live electrical connections, and the like. The enclosure can include four supporting feet, located on the underside of the enclosure, to provide a clearance of about 0.75 inches or more between the underside of the enclosure and the table top. The enclosure can include a recessed area with access to external accessory connections such as the display port, the Ethernet port, the 4 USB ports, and the like.

Referring now to the cooling sub-system included in the PCR system, an air intake can be provided in the front of the unit and an air exhaust can be provided in the rear portion of the top of the unit. Intake air can pass through the air intake and through a filter element (e.g., a removable and washable filter element). The cooling sub-system can maintain an interior air temperature (e.g., the temperature as is measured at the surface of the reagent strips, such as the reagent strips numbered 1, 12, and 24, at the surface of the PCR cartridges, and the like) about 10° C. higher, or less, than the ambient air temperature. The cooling subsystem can maintain the internal air temperature at or below about 32° C. One or more cooling fans included as part of the cooling subsystem may require about 5.7 Watts, or less, of power per fan.

In some embodiments, the system can include covers on internal subassemblies (with the exception of the gantry). The covers can be cleanable with a 10% bleach solution applied with a soft cloth without significant degradation. The covers can supply a safety barrier between a user and the electronic and moving mechanical assemblies included in the system. The covers on the internal subassemblies can be designed to maximize cooling of the internal subassemblies by maximizing airflow under the covers and minimizing airflow above the covers. The covers can be removable by a service technician and can match the color and texture of the enclosures.

In some embodiments, the system can be designed to operate within a temperature range of about 15 X to about 30 X and in a non-condensing relative humidity range (e.g., about 15% to about 80% relative humidity). The analyzer can be designed to perform without damage after exposure to storage at no less than -20° C. for 24 hours or less, storage

86

at no greater than 60° C. for 24 hours or less, and/or storage at about 50,000 feet or less (e.g., 3.4 inches of Hg) for 24 hours or less. The system can be designed with provisions to prevent motions that would damage the instrument during shipping. It can conform to the shipping standards set forth in ASTM D 4169-05DC 12 and can be designed to allow the baseplate to be securely mounted to a shipping pallet. The racks and the enclosure of the instrument are designed not to degrade or be damaged by daily cleaning with a 10% bleach solution. The power to subassemblies of the system can be supplied by internal power supplies. Exemplary power supplies can receive, as input, about 1590 watts at about 90 to about 264 Vac at between about 47 and about 63 Hz and supply about 1250 watts of output to the subassemblies.

In some embodiments, the system can include a power switch (e.g., a rocker-type switch), located on the right side of the instrument, one or more interface components, and/or one or more interface ports. For example, the system can include an LCD display monitor that is 15 inches, has 1280x1024 pixel resolution and 16-bit color. The system can also include other display monitors such as ones with increased size, resolution, and/or color depth. The LCD display can be connected to the system via a VGA connection. The system can include a white, 2 button USB mouse, a white USB keyboard, a black SJT power cable, and an un-interruptible power supply, with feedback through USB. The system can also include a USB color printer, 2 USB cables (e.g., one for the printer and one for the UPS). The system can include exemplary interface ports, such as, 4 USB ports (e.g., to connect to a pointing device, printer, keyboard, UPS, LIS), 1 VGA port (e.g., for connection to the LCD display), and 1 Ethernet port (e.g., for PC connectivity) located on the left side of the enclosure. An IEC/EN 60220-11C14 power port can be included on the right side of the enclosure.

In some embodiments, the system can include features directed at increasing the safety of a user. For example, door interlocks can be included to prevent user access while the gantry is in motion and/or while other non-interruptible processes are underway. The system can be designed to minimize or eliminate the presence of user-accessible dangerous corners and/or edges on the instrument and designed such that metal parts are properly electrically grounded. Sheet metal or plastic covers can be included over mechanical and electrical components as necessary to protect a user from moving parts and/or live electrical parts and to protect the electronics and motors included in the system from, for example, spills.

Example 19: Exemplary Optics

Described herein are exemplary specifications related to the design of optics used in a PCR Analyzer and/or System. Additional information related to the PCR System is described elsewhere herein. The optical detection system included in the PCR System can be a 12-lane two-color detection system for monitoring real-time PCR fluorescence from a 12-lane microfluidic PCR cartridge. The system can include excitation lights (e.g., blue and amber LED light sources), one or more band pass filters, and one or more focusing lenses. The emitted fluorescence light from the PCR reactor (e.g., included in the microfluidic cartridge) is captured through a pathway into a focusing lens, through a filter, and onto a photodiode. Included in the system, for each PCR lane, are dedicated, fixed individual optical elements for each of the two colors interrogated.

US 10,632,466 B1

87

In some embodiments, the limit of detection is 20 DNA copies per reaction of input PCR reaction mix with a minimum signal to base value of 1.15. The 2 color fluorescence system can be used with, for example, FAM (or equivalent) and Cal Red (or equivalent). The system can have the ability to collect fluorescence data in about 100 ms to about 600 ms at the maximum rate of one data point every about two seconds. When collecting data from a PCR lane, LEDs in adjacent lanes increase the signal in the lane being sampled by less than about 1% (e.g., 0.5%). The noise of the detection can be less than about 1% of the maximum signal. The lane-to-lane fluorescence variability with a fluorescence standard (e.g., part #14000009) can be within Cv of 30% for both PAM and Cal Red, when measured using the dark-current-corrected-fluorescence-slope. The average dark current-corrected-fluorescence-slope for the optical block with 12 lanes can be between about 30 mV to about 90 mV/(%blue LED power) for FAM using the fluorescence standard (Part #14000009). The average dark current-corrected-fluorescence-slope for the optical block with 12 lanes should be between about 75 mV to about 300 mV/(% amber LED power) for Cal Red using the standard fluorescence cartridge (Part #14000009). The average excitation power for each channel can be independently varied by software from about 5% to about 100%. There may be no source of light activated inside the reader to affect the fluorescence reading. In some embodiments, turning room lights on or off does not affect the optical readings.

In some embodiments, the system can include an optical block with 12 repeats of 2-color fluorescence detection units at a pitch of about 8 mm. The optical detection block can be positioned on top of the microfluidic cartridge, with excitation and emission travelling through the PCR windows of the microfluidic cartridge. The apertures of the optical block can align with the PCR reactor within about +/-200 microns. An optical electronics board containing the LEDs and Photodetectors can be mated flush with the top of the optics block with each of the photodetectors recessed into the bores of its corresponding optical lane. When the microfluidic cartridge is installed in the system, the optical block can be used to deliver a force of about 20 to about 30 lbs. over the active area of the microfluidic cartridge with an average pressure of at least about 1 psi.

The optical block can be made of aluminum and surfaces present in the optical path lengths can be anodized black, for example, to minimize auto-fluorescence as well as light scattering. An aperture plate having 12 slits, each slit about 10 mm in length and 1 mm wide, can be used, for example, to limit the size of the excitation light spots as well as reduce background fluorescence. The thickness of the optics block can be about 1.135+/-0.005 inches. The bottom surface of the optics block can be planar within +/-1 mil to provide uniform pressure over the micro fluidic cartridge. The apertures should be kept clean and free of debris during manufacturing of the optics block and assembly of the optics block into the system.

In some embodiments, the system can include excitation optics with an angle of excitation path equal to 55+/-0.5 inches with respect to normal of the PCR cartridge surface. One exemplary arrangement of optical elements in the excitation path, in order, is LED, lens, filter, aperture, and PCR sample. The system can use a Plano-convex excitation lens (e.g., PCX, 6x9, MgF2TS) oriented with the flat side toward the PCR sample. Included in the optics are one or more excitation paths with tapers that can be designed such that the lens and filter can be placed inside the bore to provide a light spot bigger than the aperture plate. The

88

location of the LED and the sample can be fixed as the design can include a fixed available optical block thickness. The location of the lens and the filter can be determined to provide a excitation spot size of about 6 mm along the length of a PCR lane. The excitation optics can include an LED such as Luxeon Part #LXK2-PB 14-NO0 (e.g., for FAM excitation) that includes a center wavelength of about 470 nm (blue) with a half band width of about 75 nanometers, or less (e.g., for FAM excitation). The excitation optics can also include an LED such as Luxeon Part #LXK2-PL12-Q00 (e.g., for Cal Red excitation) that includes a center wavelength of 575 nm (amber) with a half band width of about 75 nanometers, or less (e.g., for Cal Red excitation). The LEDs used in the excitation optics can remain stable for about 5 years or more or about 10,000 cycles.

The system can include emission optics with an angle of emission path equal to about 15+/-0.5 inches with respect to normal of the PCR cartridge surface. One exemplary arrangement of optical elements in the emission path, in order, is PCR sample, aperture, filter, lens, and photodetector. The emission lens can be plano-convex (e.g., PCX, 6x6 MgF2TS) with the flat side toward the photodetectors. The emission optics can include one or more bores, for the emission path, with tapers that can be designed so as to maximize detected light while enabling snug placement of the filters and lenses. The location of the photodetectors with respect to the sample can be fixed as the design can include a fixed available optical block thickness. The location of the lens and the filter can be determined so as to provide an emission spot size of 6 mm along the length of a PCR lane. An exemplary photodetector that can be used in the emission optics is the Hamamatsu Silicon Photodetector with Lens, S2386-18L.

In some embodiments, the system can include one or mote filters with diameters of about 6.0+/-0.1 mm, thicknesses of about 6.0+/-0.1 mm, clear apertures with diameters of less than or equal to about 4 mm. The filters can include a blackened edge treatment perforated prior to placement in a mounting ring. If present, the mounting ring can be metal and anodized black. The filters can be manufactured from optical glass with a surface quality that complies with F/F per Mil-C-48497A, an AOI of about 0 deg, a 1/2 cone AOI of about +8 deg. and can be humidity and temperature stable within the recommend operating range of the system. An exemplary filter can be obtained from Omega Optical BATTLEBORO, VT. 05301.

The system can include one or more FIFC Exciter Filters (e.g., PN 14000001) with an Omega part number 481AF30-TED-EXC (e.g., drawing #2006662) used, for example, in FAM excitation. These filters can have a cut-on wavelength of about 466+/-4 nm and a cut-off wavelength of about 496 +0/-4 nm. The transmission of filters of this type can be greater than or equal to about 65% of peak. These filters can have a blocking efficiency of greater than or equal to OD4 for wavelengths of ultraviolet to about 439 nm, of greater than or equal to OD4 for wavelengths of about 651 nm to about 1000 nm, of greater than or equal to OD5 for wavelengths of about 501 nm to about 650 nm, and of greater than or equal to OD8, in theory, for wavelengths of about 503 nm to about 580 nm.

The system can include one or more Amber Exciter Filters (e.g., PN 14000002) with a part number 582AF25-RED-EXC (e.g., drawing #2006664) used, for example, in Cal Red excitation. These filters can have a cut-on wavelength of about 569+/-5 nm and a cut-off wavelength of about 594+/-5 nm. The transmission of filters of this type can be greater than or equal to about 70% of peak. These filters can

have a blocking efficiency of greater than or equal to CDS, in theory, for wavelengths of about 600 nm to about 700 nm.

The system can include one or more FITC Emitter Filters (e.g., PN 14000005) with a part number 534AF40-RED-EM (e.g., drawing #2006663) used, for example, in FAM emission. These filters can have a cut-on wavelength of 514+/-2 nm and a cut off wavelength of 554+/-5 nm. The transmission of filters of this type can be greater than or equal to about 70% of peak. These filters can have a blocking efficiency of greater than or equal to CDS for wavelengths from ultraviolet to about 507 nm, of greater than or equal to OD8, in theory, from about 400 nm to about 504 nm, and of greater than or equal to OD4 avg. from about 593 nm to about 765 nm.

The system can include one or more Amber Emitter Filters (e.g., PN 14000006) with a part number 627AF30-RED-EM (e.g., drawing #006665) used, for example, in Cal Red emission. These filters can have a cut-on wavelength of 612+5/-0 nm and a cut-off wavelength of 642+/-5 nm. The transmission of filters of this type can be greater than or equal to about 70% of peak. These filters can have a blocking efficiency of greater than or equal to OD8 for wavelengths from ultraviolet to about 605 nm, of greater than or equal to OD8, in theory, from about 550 nm to about 600 nm, and of greater than or equal to OD5 avg. from about 667 nm to about 900 nm.

Example 20: Exemplary 3-layer Cartridge

Described herein are exemplary specifications used to design and assemble the microfluidic cartridge as well as exemplary instructions on the use of the cartridge in, for example, the system described herein. In some embodiments, the cartridge can have a maximum limit of detection equal to 20 copies per reaction volume (e.g., 20 copies/4µl), with a target detection of 10 copies per reaction volume. The cartridge can perform 45 reaction cycles in 40 minutes or less (e.g., 45 cycles in 40 minutes, 45 cycles in 20 minutes, 45 cycles in 15 minutes, or the like). The cartridge can utilize two color detection using, for example, the FAM (or equivalent) and CAL RED (or equivalent) fluorescent dyes. Results obtained using the cartridge have been compared with the results obtained using standard real-time PCR instruments.

In some embodiments, the Cartridge can be a one-time use, disposable cartridge that can be disposed of according to typical laboratory procedures. The cartridge can be 4.375 inches long and 2.800 inches wide, with a thickness of 0.094+/-0.005 inches. The cartridge can include features that allow the cartridge to interface with, for example, the system described herein. Exemplary interfacing features include PCR channel walls and the top of the micro-substrate over the PCR channel that are well polished (SF1 A1/A2/A3), enabling easy transfer of excitation and emission light between the PCR reactor (e.g., contained in the cartridge) and the detection system (e.g., the analyzer). The cartridge can include a thermal interface, located on the bottom of the cartridge, for interfacing with the analyzer. The thermal interface can have a thin laminate (e.g., less than 150 microns thick, 100 microns thick, or the like) to encourage heat transfer from the heater wafer to, for example, the PCR channels of the cartridge.

The cartridge can include one or more mechanical interfaces with, for example, the analyzer. For example, the cartridge can have a notch in one or more of the corners that can mate with a corresponding shape on the heater module of the analyzer. The notch and corresponding shape, can enable the cartridge to be placed only one way in the tray of,

for example, the system described herein. In some embodiments, the cartridge has a single notch in one of the corners, with the remaining three corners having a minimum radius of 1 mm to facilitate placement of the cartridge in the analyzer. During use (e.g., when placed in a system described herein and performing a function such as PCR), the cartridge can be pressed, on one side, by the optics block, against the heater wafer (positioned against the opposite side), with a pressure of about 1 psi or greater (e.g., 0.99 psi 1.2 psi, or the like). When located in the tray of the analyzer, the cartridge can have an alignment slop of +/-200 microns to enable a user to easily place and remove the cartridge from the analyzer tray. The cartridge can have two ledges, that are each 1 mm wide and located along the two long edges of the cartridge, to enable the heating surface to extend below the datum of the tray.

In some embodiments* the cartridge can have the following functional specifications. The cartridge can include an inlet hole that is, for example, cone-shaped with a height of 1 mm from the top surface of the cartridge. The cone can have an inner diameter of 3 mm at the top of the cone and can taper down to a diameter that matches the width of a microchannel (e.g., in inlet channel) that the inlet cone is fluidly connected to. The inlet channel can fluidly connect the inlet hole to a PCR reactor that has an interior volume of, for example, about 4.25 µl to 4.75 µl (e.g., 4.22 µl, 4.5 µl, 4.75 µl, or the like). An outlet microfluidic channel can fluidly connect the PCR reactor to an overflow chamber. The cartridge can also include an outlet vent hole.

The input PCR sample (e.g., a reaction mixture) can be between about 6.0 and 7.0 µl per PCR lane (e.g., 5.9 µl per lane, 6.4 µl per lane, 7.1 µl per lane, or the like) and can be introduced into the cartridge through the inlet hole by, for example, a pipette. The reaction mixture can be transported, via the inlet channel to the PCR reactor where the reaction mixture can be isolated (e.g., sealed off by valves) to prevent evaporation or movement of the reaction mixture during thermocycling. Once the mixture is sealed inside the chamber, the analyzer can initiate multiplexed real-time PCR on some or all of the reaction mixture (e.g., 4.5 µl, an amount of fluid equal to the inner volume of the reaction chamber, or the like).

The microfluidic substrate of the cartridge can include one or more of the following specifications. The material of the microsubstrate can be optically clear (e.g., have about 90% or greater optical transmission, be 3 mm thick, comply with ASTM D1003, and the like), have auto-fluorescence that is less than that emitted by 2 mm thick ZEONOR 1420R, and have a refractive index of about 1.53 (ASTM D542). The material of the microsubstrate can be amenable to the injection molding of features required for the microfluidic network of the cartridge. The material is preferably compatible with all PCR agents and can withstand temperatures of up to about 130° C. for about 5 minutes or more without yielding or melting. The cartridge can include fiducials, recognizable by HandyLab manufacturing equipment, located in one or more (preferably two) of the corners of the substrate. The cartridge can include fluidic components (e.g., microchannels, valves, end vents, reagent inlet holes, reaction chambers, and the like) necessary to perform the functions of the cartridge (e.g., PCR).

Additional features of the substrate material can include one or more of the following. Minimum clearances of about 1 mm can be designed between functional features to ensure sealing success (e.g., to the analyzer), and to allow simplified fixturing during assembly. The cartridge can include dogbones under small fluid path ends to, for example,

US 10,632,466 B1

91

increase mold life. The bottom of the micro tool surface can be roughened (e.g., by vapor hone, EDM, or the like). The substrate material can be capable of adhesion by a label.

In some embodiments, the sealing tape used in the cartridge can include one or more of the following specifications. Laminate can be easily applied to the bottom of the microfluidic substrate. Material of the laminate is preferably pin-hole free. The material and adhesive is preferably compatible with the PCR reaction chemistries. The laminate material and glue used should not auto-fluoresce. The material can withstand up to 130° C. for 3 minutes without losing adhesion, yielding, melting, or causing undue stresses on the cartridge. Bubbles should not form in the adhesive layer upon heating (e.g., to 130° C. for 5 minutes) after application to the microsubstrate. The laminate should be less than 5 mills thick to, for example, enable rapid heat transfer.

The high temperature wax included in the cartridge can have the following characteristics. The wax should have a melt point of about 90+/-3° C. (e.g., 87° C., 90° C., 93.1° C., or the like), be biocompatible with PCR reactions, have wettability with microsubstrate material, and have a melt viscosity range, for example, of about Viscosity at 100° C.=20 mm²/s and Hardness at 25° C.=8 dmm. The main label of the cartridge can have the following characteristics. It can have a thickness of 2-4 mils, have suitable bondability to micro features and seal around the valves, include cuts for one or more PCR windows, and a tab (free from adhesive) for aiding in removal of the cartridge from the analyzer. The main label can also have abrasion resistance on the top surface, and be printable. The main label can have an upper and lower alignment pattern for the label to completely cover the valve holes for proper operation of the valves.

The cartridge can include a barcode label applied to the top of the cartridge that is readable by a barcode reader (e.g., the barcode reader included in the analyzer) while the cartridge is installed in the analyzer. The barcode label can include the product name, lot #, expiration date, bar code (2D) and may be printed on. In addition, or in the alternative, a barcode may be applied directly to the main cartridge label using a laser or inkjet type printer.

The packaging that the cartridge is included in can include one or more of the following: package label, carton, carton label, and/or operating instructions. The packaging can be printed on or label attachable, placed inside of a plastic bag, shrink/stretch wrap bag, or the like, and can be stacked in groups of 24. The cartridge bagging without a critical seal should be kept free from dust contamination,

The cartridge can include one or more valves (e.g., temperature controlled, wax-containing valves) for starting, stopping, and/or controlling the flow of material inside the cartridge. The wax contained in the valves can be free of trapped air bubbles that have a diameter greater than half the width of the valve channel. The valve channel can have an air pocket. The wax may not intrude into the fluid path prior to activation. The wax can be filled to the start of the flare to the fluid path.

The cartridge can include micro channels and holes such that the holes are of a size and shape to enable easy, leak-free interfacing with a 175 µl pipette tip. In some examples, the holes size is between about 200 µm and about 4000 µm in diameter. The microchannels can be between about 50 µm and about 1500 µm wide and between about 50 µm and 1000 µm high.

The cartridge can include valves for controlling the flow of fluid within the cartridge (e.g., through the microchannels, reactor chambers, and the like). The valve edges, steps, and general geometry can be designed to encourage exact

92

flow and/or stoppage required during wax load. The valve geometry can be designed to accommodate limitations of wax dispensing equipment (e.g., +/-25% of 75 nL volume). In some embodiments, step down air chambers on the valves are funnel shaped to aid wax loading and the remaining geometry diminishes from the bottom of the funnel to the end point where the wax stops. The path where the valves are to flow into and block, during use, can be narrow enough (e.g., 150-200 microns wide and deep) and have enough length to effectively seal when the valves are activated during use. The valve wax temperature can be about 90° C. When in use to block a portion of a microchannel, the valves can seal to prevent evaporation of fluid and/or physical migration of fluid from the PCR reactor during thermocycling.

The cartridge can include one or more PCR regions for performing PCR on a sample. The channel in the PCR region (e.g., PCR reactor) can be designed such that the temperature of the contents of the channel remain uniformly within about 2° C. of the anneal temperature. The channel walls can have a polish of SR1 A1/A2/A3.

In some embodiments, the cartridge is designed to be able to perform diagnostic tests within a temperature range of about 59° F. to about 86° F. (about 15° C. to about 30° C.) and a humidity range of about 15% relative humidity to about 80% relative humidity. The cartridge is designed to be safe and functional when used indoors, used at an altitude of 2000 m or less, and used under non-condensing humidity conditions (e.g., maximum relative humidity of 80% for temperatures up to 31° C. decreasing linearly to 50% relative humidity at 40° C.).

In use, PCR product produced in the cartridge can remain in the used cartridge to, for example, minimize the likelihood of cross contamination. The cartridge can be designed such that a 4 foot drop of the cartridge, while in its packaging, will not damage the cartridge. The cartridge is designed to perform without damage after exposure to the following conditions. The cartridge should be stored at 4° C. to 40° C. for the rated shelf life. Exposure to temperatures between -20° C. and 4° C. or 40° C. and 60° C. should occur for no longer than 24 hours. The cartridge can withstand air pressure changes typical of air transport.

The cartridge can be labeled with the following information (e.g., to identify the cartridge, comply with regulations, and the like). The label can contain a "Research Use Only" label, if applicable, and a CE mark, if applicable. The label can contain the company name and logo (e.g., Handylab®), a part number (e.g., 55000009), a part name (12x Cartridge-nonvented), a lot number (e.g., LOT 123456), an expiration date (e.g., Jun. 2015), space for writing, a barcode according to barcode specifications (described elsewhere), and/or "Handylab, Inc., Ann Arbor, Mich. 48108 USA".

The cartridge can be included in a carton that can contain information such as, a part number (e.g., 55000009), a part name (12x Cartridge-nonvented), a quantity (e.g., 24), a lot number (e.g., LOT 123456), an expiration date (e.g., Jun., 2015), an optional UPC code, "Manufactured by Handylab, Inc., Ann Arbor, Mich. 48108 USA", a carton label to state storage limits, a CE mark (if applicable), and/or an AR name and address.

The cartridge packaging can include paper wrap to secure multiple cartridges together and clean package fill to prevent damage, for example, from vibration. The cartridge shipping carton can include features such as, compliance to ASTM 6159, carton may be stored in any direction, refrigeration or fragile labeling of the carton may not be required, and

US 10,632,466 B1

93

additional cold packs may not be required. The shelf life of the cartridge is 12 months or more.

The cartridge can comply with IEC 61010 (NRTL tested) and an FDA listing may be required for clinical distribution. Cartridges used in a clinical lab device may meet all quality system requirements. Cartridges used for research only in a commercial device may meet all HandyLab quality system requirements. Cartridges for research use only (Alpha or Beta testing) may be design/manufacturing traceable to a DHR (manufacturing record).

The foregoing description is intended to illustrate various aspects of the present inventions. It is not intended that the examples presented herein limit the scope of the present inventions. The technology now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method of analyzing a plurality of nucleic acid-containing samples, the method comprising:

extracting nucleic acids from the plurality of nucleic acid-containing samples in a first module and amplifying the nucleic acid extracted from the plurality of nucleic acid-containing samples simultaneously in a second module using a system comprising a liquid dispenser and a bay, the first module comprising a magnetic separator and a heating assembly, wherein extracting the nucleic acids comprises:

removably receiving a housing comprising a plurality of process chambers in the bay, the plurality of process chambers maintained at a same height relative to one another as the housing is received in and removed from the bay, the plurality of process chambers aligned along a first axis when the housing is received in the bay, the bay comprising one or more complementary registration members that receive the housing in a single orientation when the housing is received in the bay, the magnetic separator of the first module positioned to apply a magnetic force to a first side of the plurality of process chambers when the housing is received in the bay, the magnetic separator comprising one or more magnets aligned along a second axis parallel to the first axis when the housing is received in the bay, the one or more complementary registration members aligning the plurality of process chambers with the magnetic separator when the housing is received in the bay, the heating assembly of the first module positioned adjacent to a second side of the plurality of process chambers opposite the first side when the housing is received in the bay, the heater assembly comprising one or more heaters aligned along a third axis parallel to the first axis when the housing is received in the bay, the one or more complementary registration members aligning the plurality of process chambers with the heater assembly when the housing is received in the bay;

moving the liquid dispenser between the plurality of nucleic acid-containing samples and the plurality of process chambers when the housing is received in the bay;

dispensing, using the liquid dispenser, at least a portion of the plurality of nucleic acid-containing samples and a plurality of magnetic binding particles into the plurality of process chambers when the housing is received in the bay;

applying a magnetic force to the first side of the plurality of process chambers using the one or more magnets of

94

the magnetic separator of the first module when the housing is received in the bay;

holding the plurality of magnetic binding particles bound to nucleic acids of the plurality of nucleic acid-containing samples against walls of the plurality of process chambers using the magnetic separator of the first module;

moving, using the liquid dispenser, a portion of a solution contained in each of the plurality of process chambers to a waste chamber;

dispensing, using the liquid dispenser, a wash buffer into the plurality of process chambers;

dispensing, using the liquid dispenser, a release buffer into the plurality of process chambers and over the plurality of magnetic binding particles in the plurality of process chambers;

heating the release buffer in the plurality of process chambers to between about 50° C. and about 85° C. using the heater assembly of the first module;

using the liquid dispenser, withdrawing liquid containing extracted nucleic acids from the plurality of process chambers; and

dispensing the nucleic acid extracted from the plurality of nucleic-acid containing samples into the second module.

2. The method of claim 1, removing the plurality of process chambers from the bay, the plurality of process chambers maintained at the same height relative to one another as the plurality of process chambers are removed from the bay; and removably receiving a second plurality of process chambers in the bay.

3. The method of claim 1, wherein the plurality of nucleic acid-containing samples are in one-to-one correspondence with the plurality of process chambers when the housing is received in the bay.

4. The method of claim 1, wherein the magnetic force is applied to the first side of the plurality of process chambers using a plurality of discrete magnets of the magnetic separator.

5. The method of claim 1, wherein holding the plurality of magnetic binding particles against walls of the plurality of process chambers comprises maintaining the one or more magnets in close proximity to the plurality of process chambers.

6. The method of claim 5, wherein maintaining the one or more magnets in close proximity to the plurality of process chambers comprises maintaining the one or more magnets between about 1 mm and about 2 mm away from the first side of the plurality of process chambers.

7. The method of claim 1, wherein holding the plurality of magnetic binding particles against walls of the plurality of process chambers concentrates the plurality of magnetic binding particles in a location inside each of the plurality of process chambers.

8. The method of claim 1, wherein the plurality of magnetic binding particles are in suspension in solutions in the plurality of process chambers when the plurality of magnetic binding particles are held against the walls of the plurality of process chambers.

9. The method of claim 8, wherein holding the plurality of magnetic binding particles against walls of the plurality of process chambers collects the suspended plurality of magnetic binding particles in a location inside each of the plurality of process chambers.

US 10,632,466 B1

95

10. The method of claim 1, wherein one pole of the one or more magnets faces toward the heater assembly and the other pole of the one or more magnets faces away from the heater assembly.

11. The method of claim 1, wherein the one or more heaters of the heater assembly comprise a heat block formed from a single piece of metal.

12. The method of claim 11, wherein the heat block is shaped to conform closely to the shape of the plurality of process chambers to increase a surface area of the heat block that is in contact with the plurality of process chambers during heating of the plurality of process chambers.

13. The method of claim 1, wherein thermal energy and magnetic energy are provided to the plurality of process chambers without moving the plurality of process chambers to a different location to perform heating or magnetic separation.

14. The method of claim 2, wherein the liquid dispenser comprises one or more dispense heads configured to accept a pipette tip.

15. The method of claim 14, wherein the liquid dispenser comprises four dispense heads and the plurality of process chambers comprises twelve process chambers, each dispense head configured to dispense a plurality of magnetic binding particles and at least a portion of one sample of the plurality of nucleic acid-containing samples into one of the twelve process chambers when the plurality of process chambers are received in the bay.

16. The method of claim 1, wherein the system comprises more than one bay, and wherein the method further comprises removably receiving a plurality of process chambers in each bay.

17. The method of claim 1, wherein the number of nucleic acid-containing samples is twelve.

18. The method of claim 1, further comprising independently detecting a plurality of fluorescent dyes at a plurality of different locations in the second module, wherein each fluorescent dye binds to a fluorescent polynucleotide probe or a fragment thereof.

19. The method of claim 18, wherein independently detecting the plurality of fluorescent dyes comprises selectively emitting light in an absorption band of the plurality of fluorescent dyes and selectively detecting light in an emission band of the plurality of fluorescent dyes.

20. The method of claim 1, wherein the plurality of nucleic acid-containing samples are extracted, amplified, and detected in less than an hour.

21. The method of claim 1, further comprising:
receiving the nucleic acid extracted from the plurality of nucleic acid-containing samples in a plurality of reaction zones; and
amplifying the nucleic acid extracted from the plurality of nucleic acid-containing samples by applying heat at one or more selected times to the plurality of reaction zones using at least one heat source.

22. The method of claim 21, wherein applying heat using the at least one heat source comprises maintaining a negligible temperature gradient across each of the plurality of reaction zones.

23. A method of analyzing a plurality of nucleic acid-containing samples, the method comprising:

extracting nucleic acids from the plurality of nucleic acid-containing samples in a first module using a system comprising a liquid dispenser, the first module comprising a bay, a magnetic separator, and a heating assembly, wherein extracting the nucleic acids comprises:

96

removably receiving a housing comprising a plurality of process chambers in the bay, the plurality of process chambers maintained at a same height relative to one another as the housing is received in and removed from the bay, the plurality of process chambers aligned along a first axis when the housing is received in the bay, the bay comprising one or more complementary registration members that receive the housing in a single orientation when the housing is received in the bay, the magnetic separator positioned to apply a magnetic force to a first side of the plurality of process chambers when the housing is received in the bay, the magnetic separator comprising one or more magnets aligned along a second axis parallel to the first axis when the housing is received in the bay, the one or more complementary registration members aligning the plurality of process chambers with the magnetic separator when the housing is received in the bay, the heating assembly positioned adjacent to a second side of the plurality of process chambers opposite the first side when the housing is received in the bay, the heater assembly comprising one or more heaters aligned along a third axis parallel to the first axis when the housing is received in the bay, the one or more complementary registration members aligning the plurality of process chambers with the heater assembly when the housing is received in the bay;

moving the liquid dispenser between the plurality of nucleic acid-containing samples and the plurality of process chambers when the housing is received in the bay;

dispensing, using the liquid dispenser, at least a portion of the plurality of nucleic acid-containing samples and a plurality of magnetic binding particles into the plurality of process chambers when the housing is received in the bay;

applying a magnetic force to the first side of the plurality of process chambers using the one or more magnets of the magnetic separator of the first module when the housing is received in the bay;

holding the plurality of magnetic binding particles bound to nucleic acids of the plurality of nucleic acid-containing samples against walls of the plurality of process chambers using the magnetic separator of the first module; moving, using the liquid dispenser, a portion of a solution contained in each of the plurality of process chambers to a waste chamber;

dispensing, using the liquid dispenser, a wash buffer into the plurality of process chambers;

dispensing, using the liquid dispenser, a release buffer into the plurality of process chambers and over the plurality of magnetic binding particles in the plurality of process chambers;

heating the release buffer in the plurality of process chambers to between about 50° C. and about 85° C. using the heater assembly of the first module;

using the liquid dispenser, withdrawing liquid containing extracted nucleic acids from the plurality of process chambers; and

dispensing the nucleic acid extracted from the plurality of nucleic acid-containing samples into a second module, the second module configured to receive a multi-lane microfluidic cartridge configured to simultaneously amplify the nucleic acid extracted from the plurality of nucleic acid-containing samples.

US 10,632,466 B1

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24. The method of claim 23, removing the plurality of process chambers from the bay, the plurality of process chambers maintained at the same height relative to one another as the plurality of process chambers are removed from the bay; and removably receiving a second plurality of process chambers in the bay. 5

25. The method of claim 23, wherein the plurality of nucleic acid-containing samples are in one-to-one correspondence with the plurality of process chambers when the housing is received in the bay. 10

26. The method of claim 23, wherein the magnetic force is applied to the first side of the plurality of process chambers using a plurality of discrete magnets of the magnetic separator.

27. The method of claim 23, wherein the one or more heaters of the heater assembly comprise a heat block formed from a single piece of metal. 15

28. The method of claim 23, wherein thermal energy and magnetic energy are provided to the plurality of process chambers without moving the plurality of process chambers to a different location to perform heating or magnetic separation. 20

29. The method of claim 23, wherein the number of nucleic acid-containing samples is twelve.

30. The method of claim 23, further comprising independently detecting a plurality of fluorescent dyes at a plurality of different locations in the second module, wherein each fluorescent dye binds to a fluorescent polynucleotide probe or a fragment thereof. 25

* * * * *

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EXHIBIT 43



US010364456B2

(12) **United States Patent**
Wu et al.

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(54) **METHOD FOR PROCESSING
POLYNUCLEOTIDE-CONTAINING SAMPLES**

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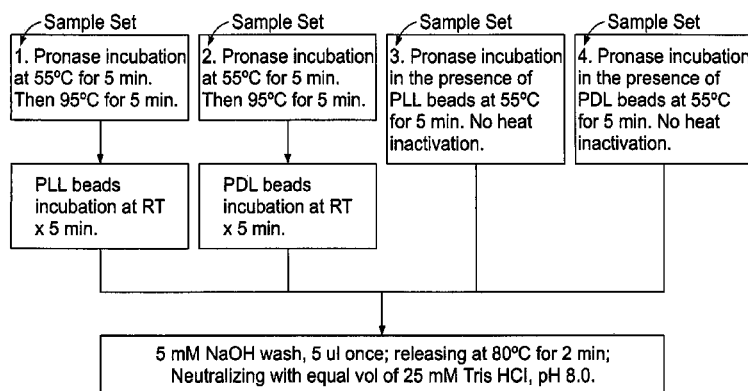
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(57) **ABSTRACT**

Methods and systems for processing polynucleotides (e.g., DNA) are disclosed. A processing region includes one or more surfaces (e.g., particle surfaces) modified with ligands that retain polynucleotides under a first set of conditions (e.g., temperature and pH) and release the polynucleotides under a second set of conditions (e.g., higher temperature and/or more basic pH). The processing region can be used to, for example, concentrate polynucleotides of a sample
(Continued)



US 10,364,456 B2

Page 2

and/or separate inhibitors of amplification reactions from the polynucleotides. Microfluidic devices with a processing region are disclosed.

20 Claims, 25 Drawing Sheets

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Page 11

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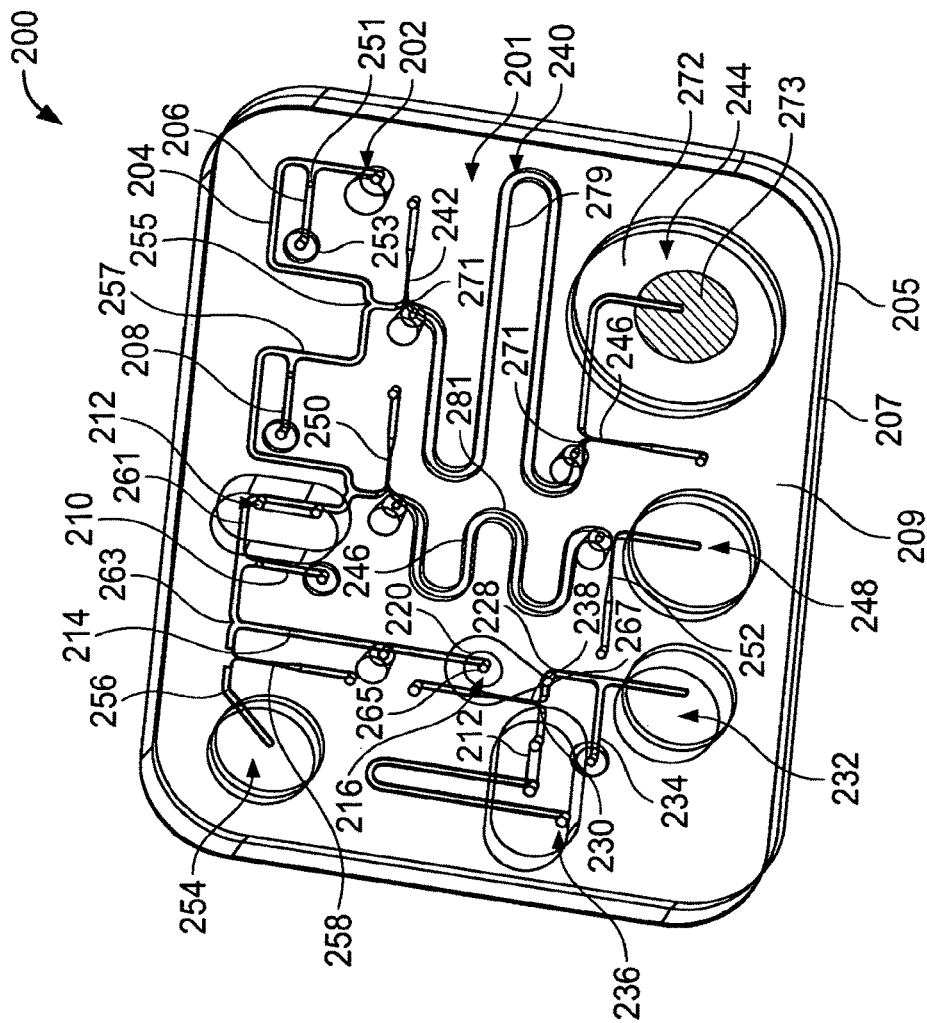


FIG. 1

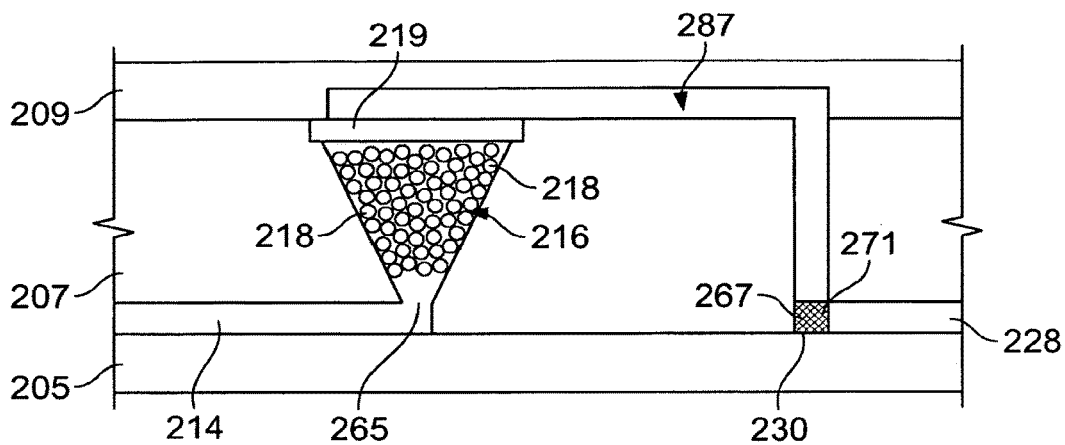


FIG. 2

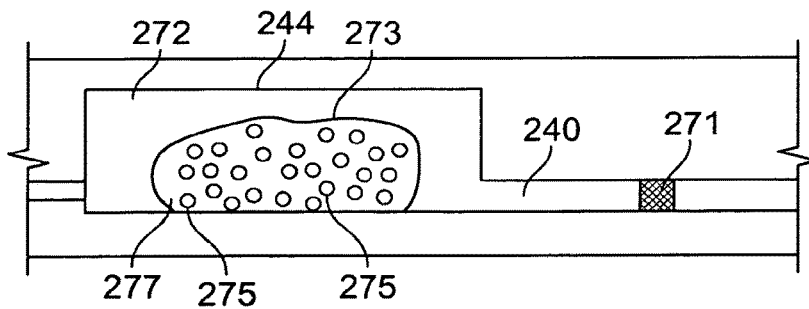


FIG. 3

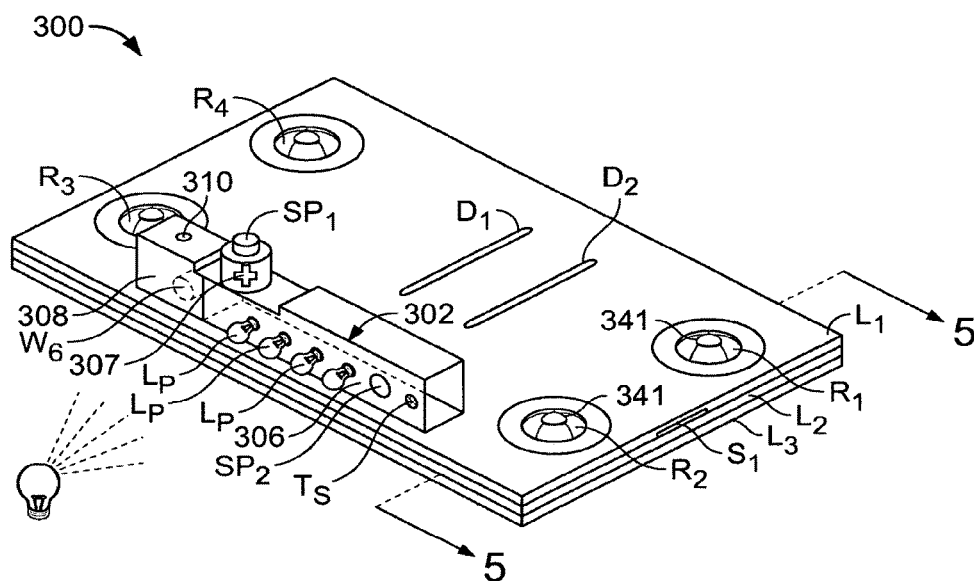


FIG. 4

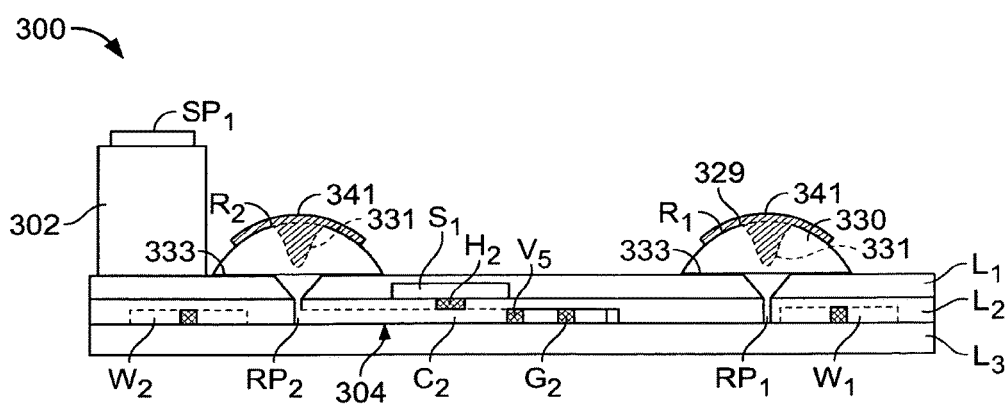


FIG. 5

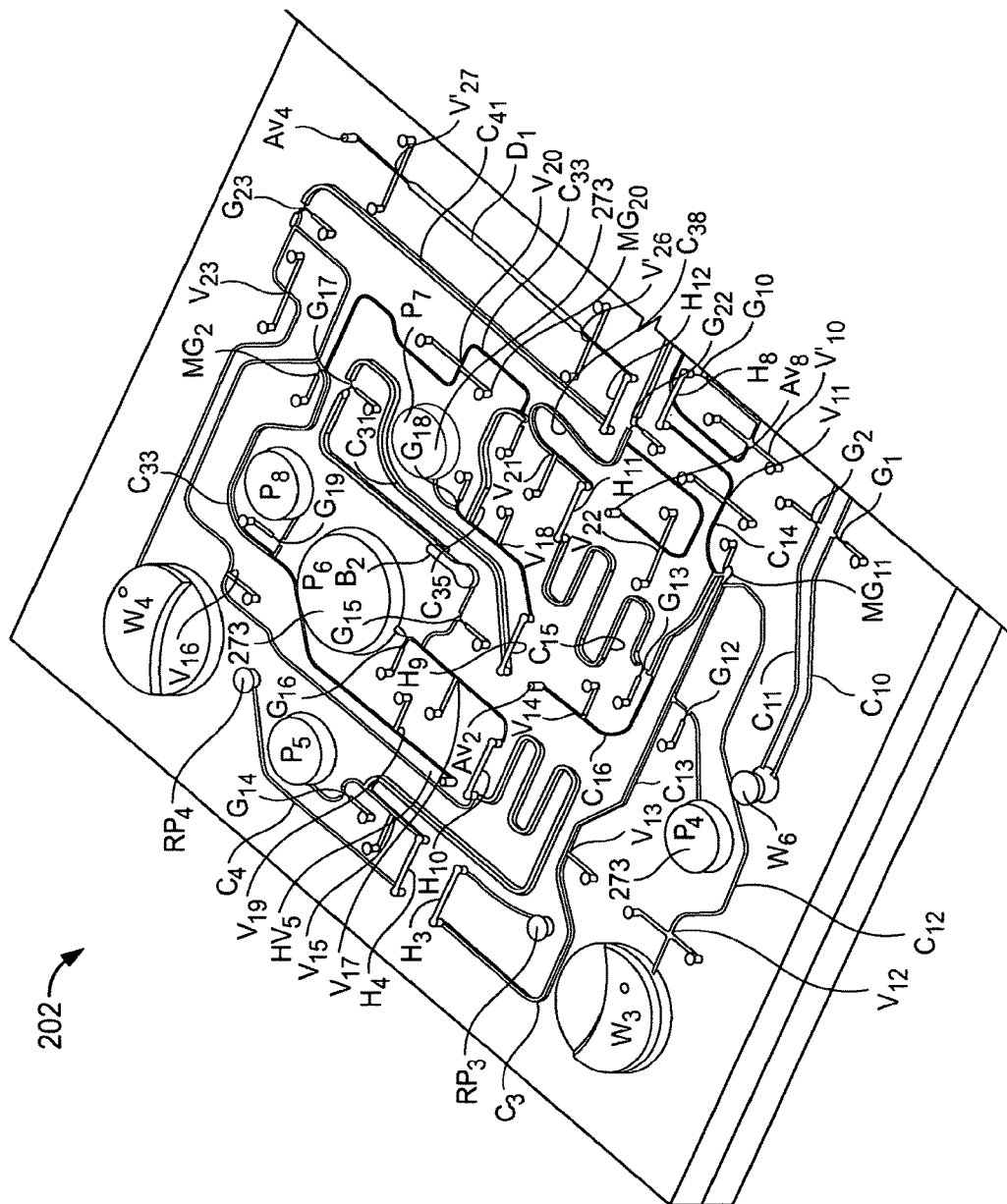


FIG. 6A

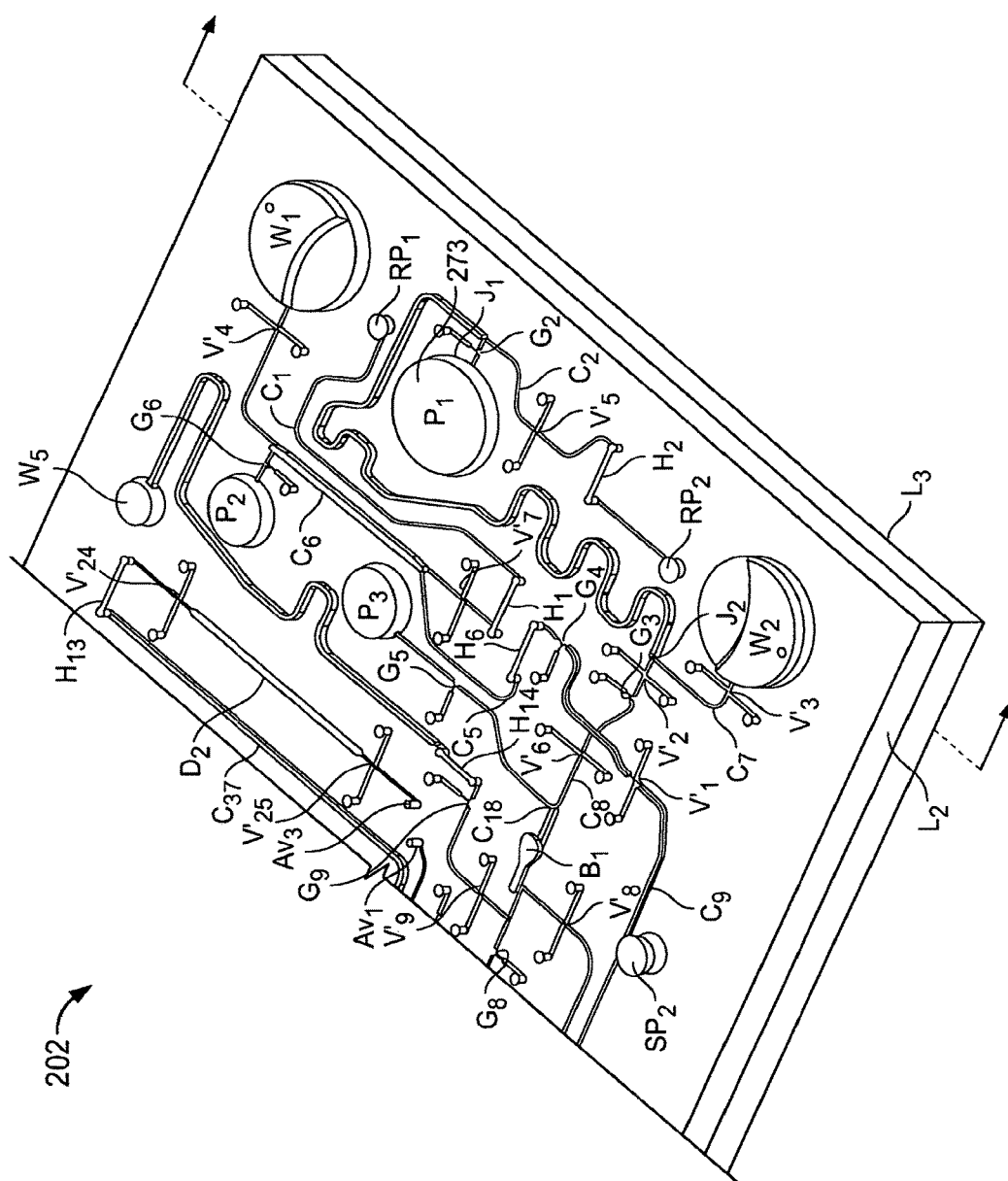


FIG. 6B

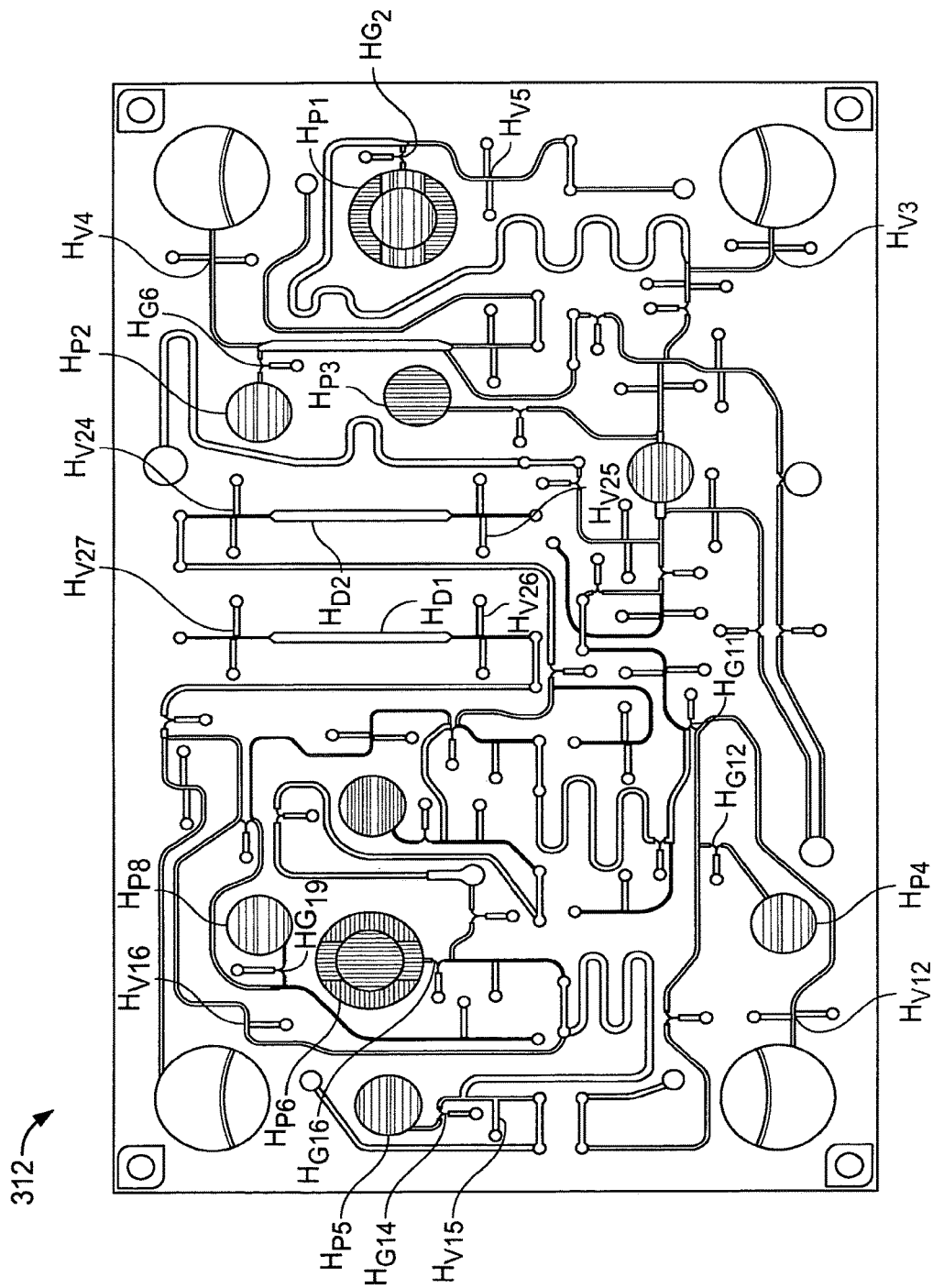


FIG. 7

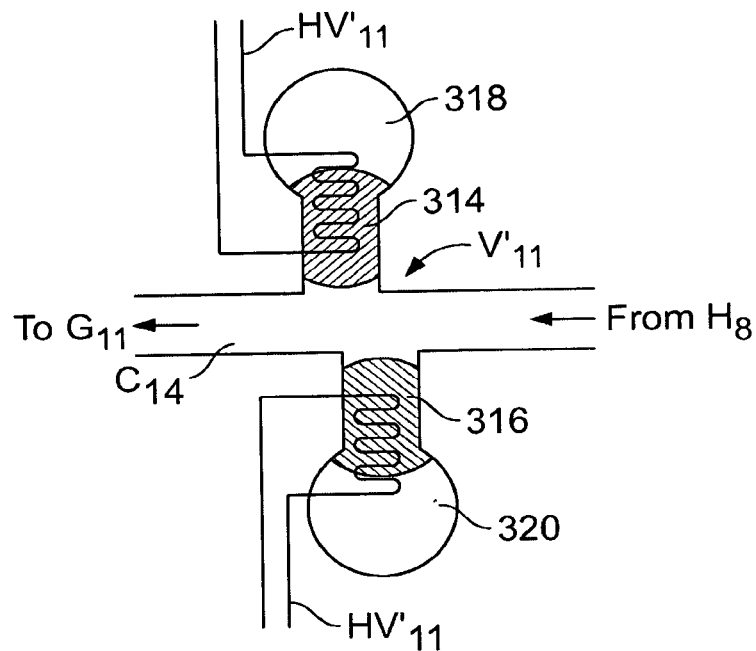


FIG. 8

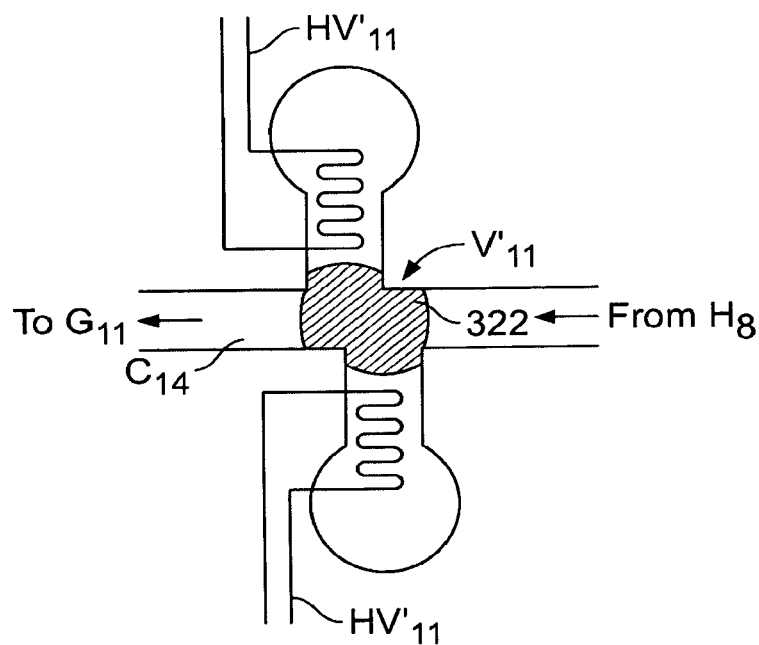


FIG. 9

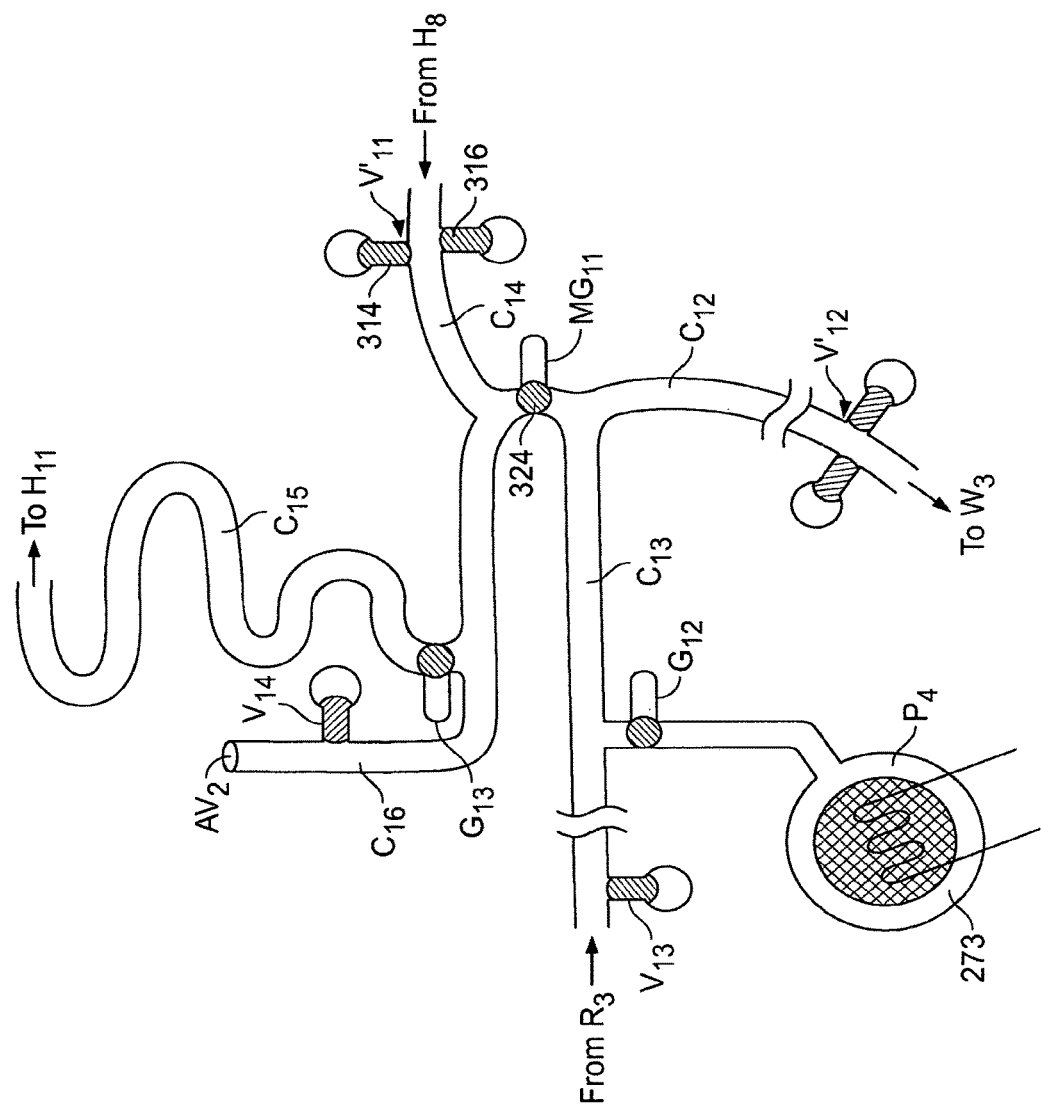


FIG. 10A

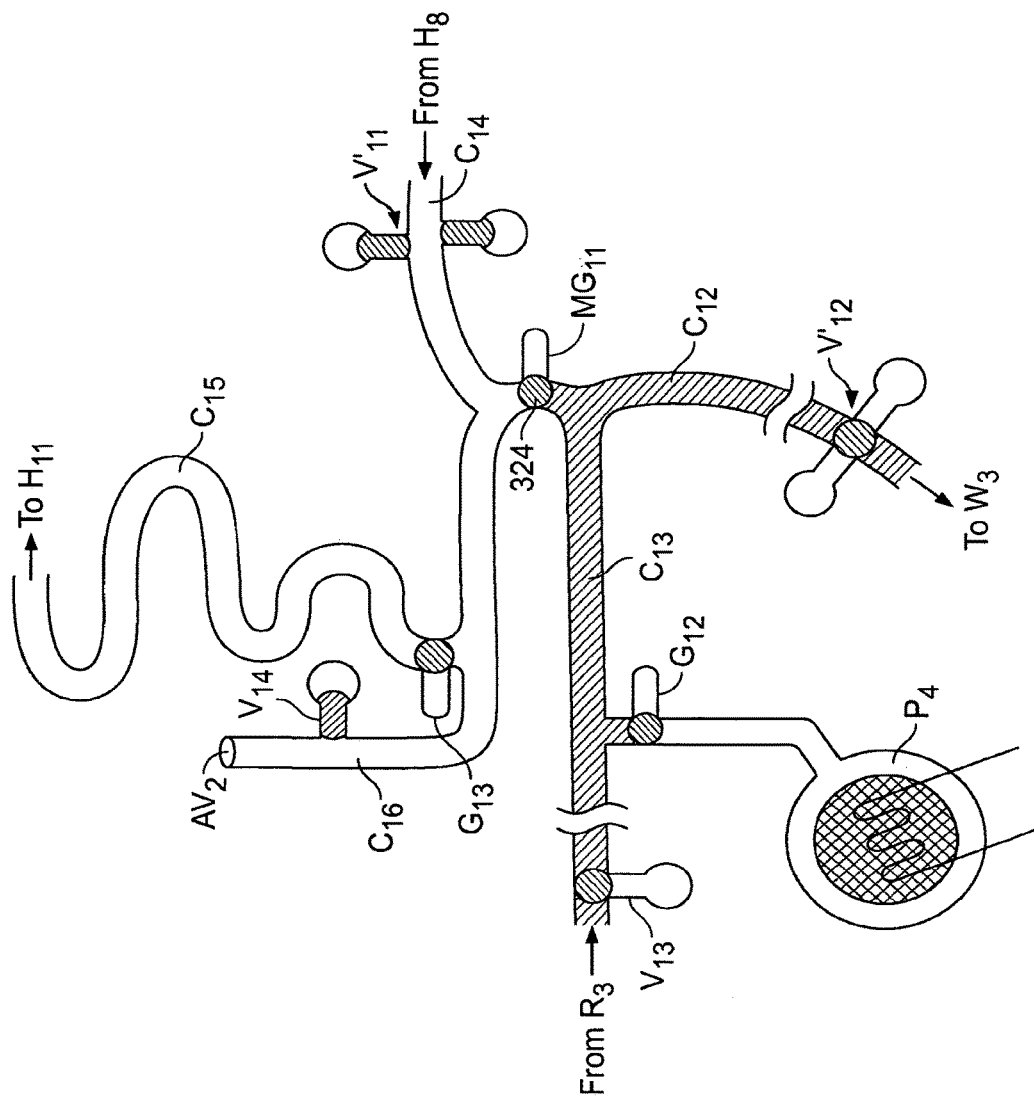


FIG. 10B

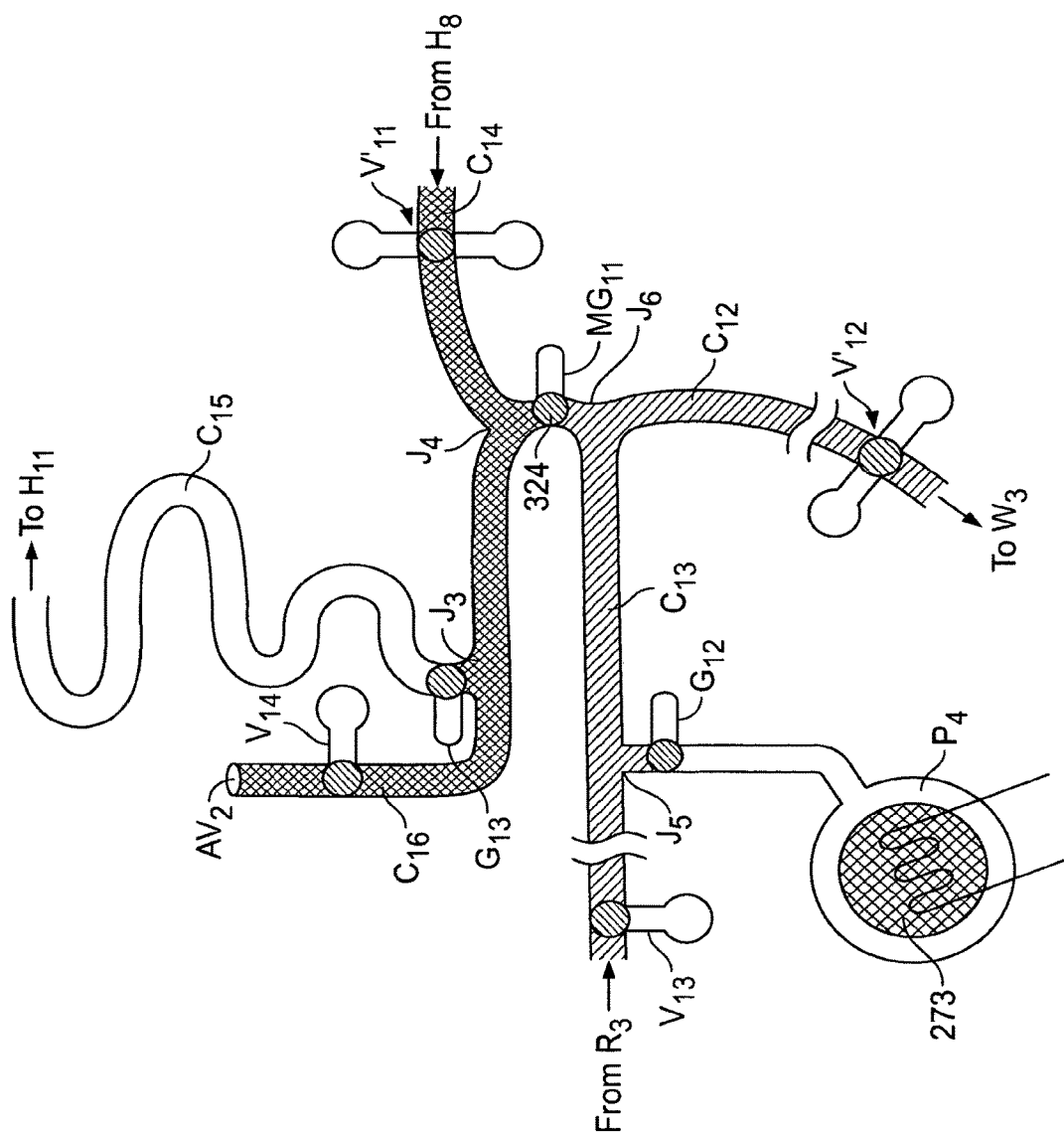


FIG. 10C

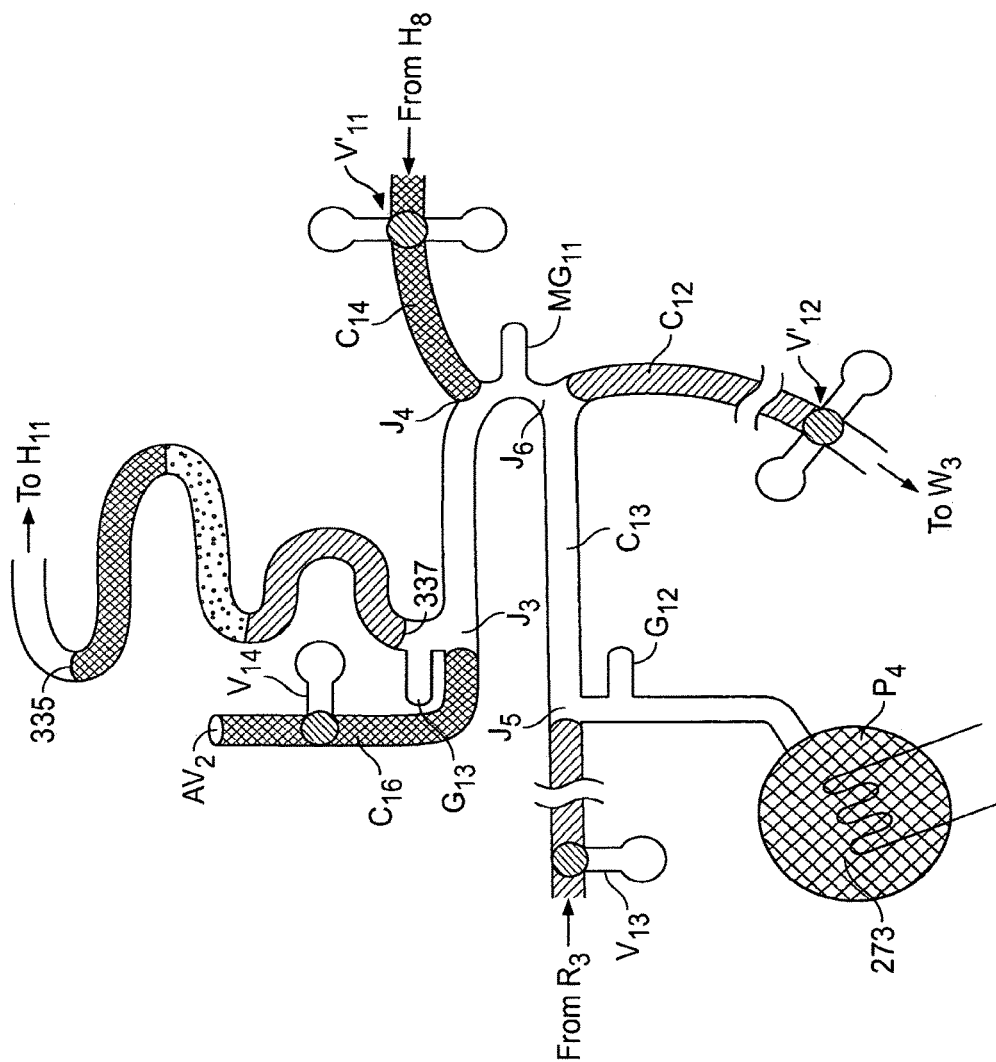


FIG. 10D

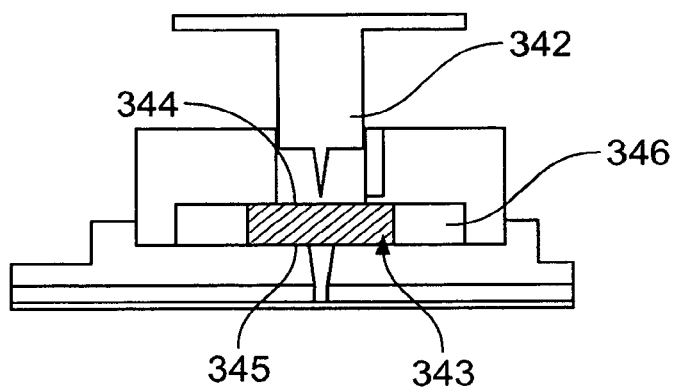


FIG. 11A

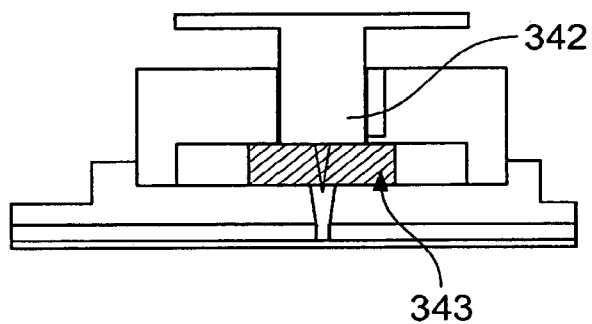


FIG. 11B

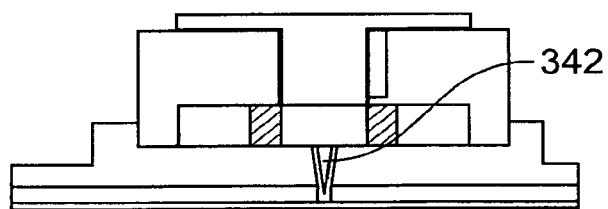


FIG. 11C

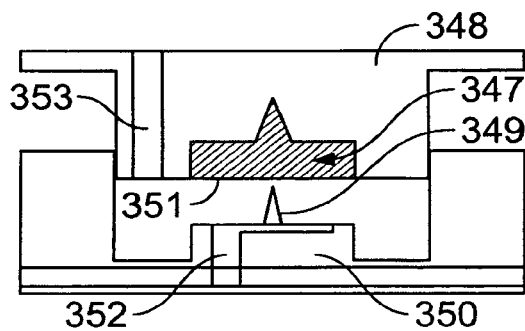


FIG. 12A

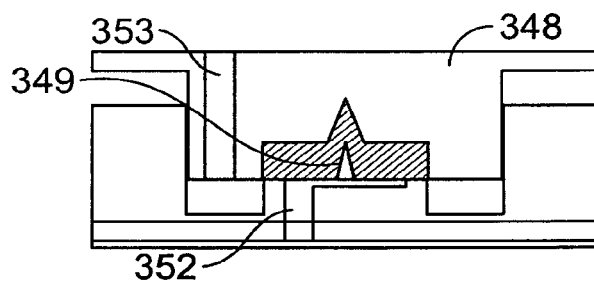


FIG. 12B

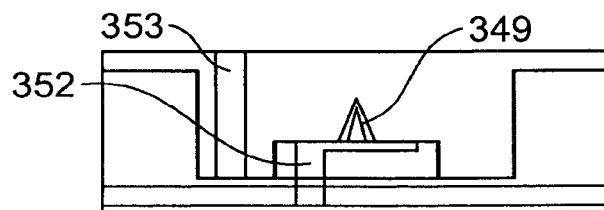


FIG. 12C

U.S. Patent

Jul. 30, 2019

Sheet 14 of 25

US 10,364,456 B2

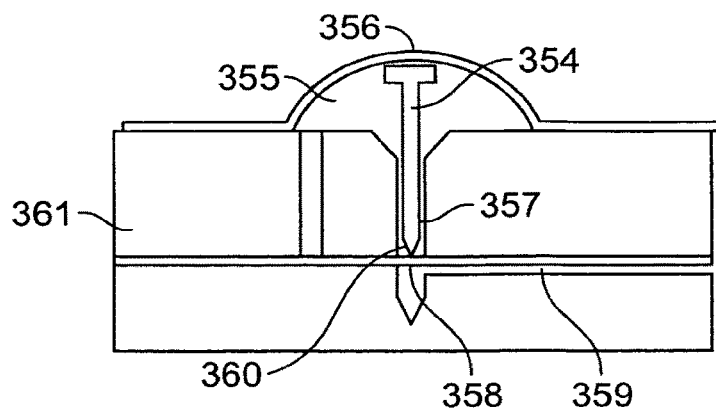


FIG. 13

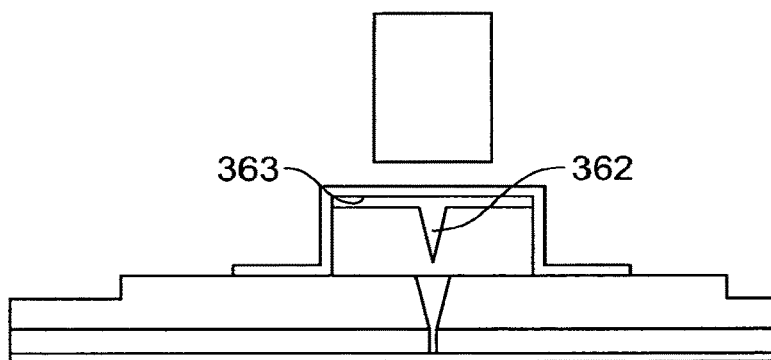


FIG. 14A

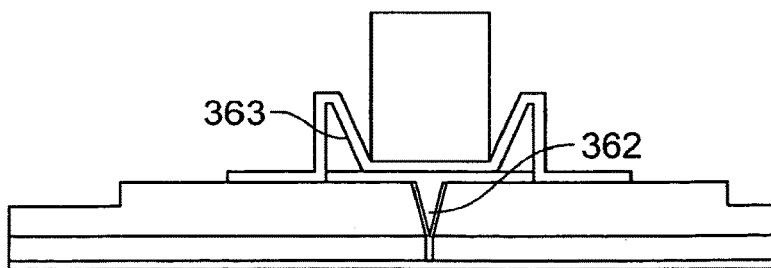


FIG. 14B

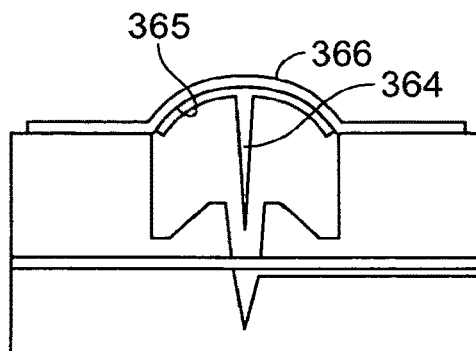


FIG. 15A

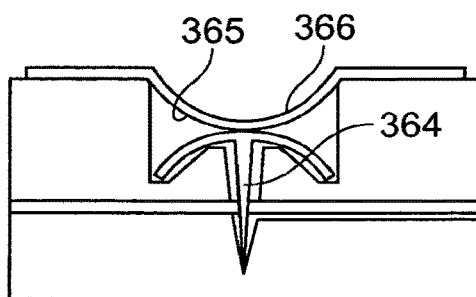


FIG. 15B

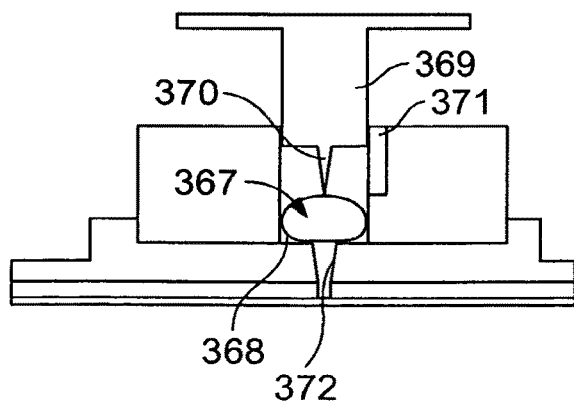


FIG. 16

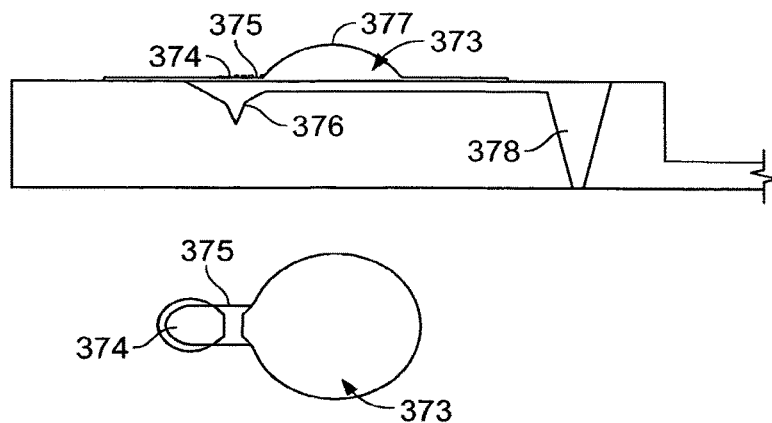


FIG. 17

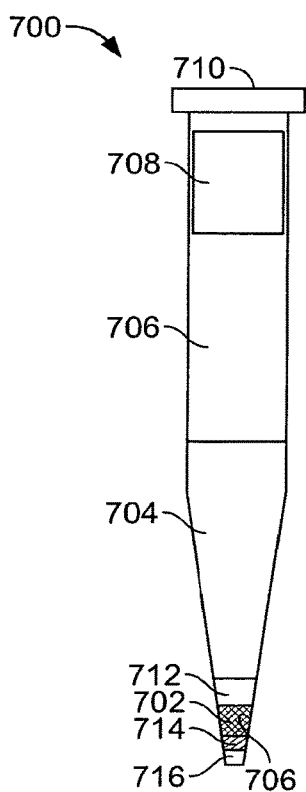


FIG. 18

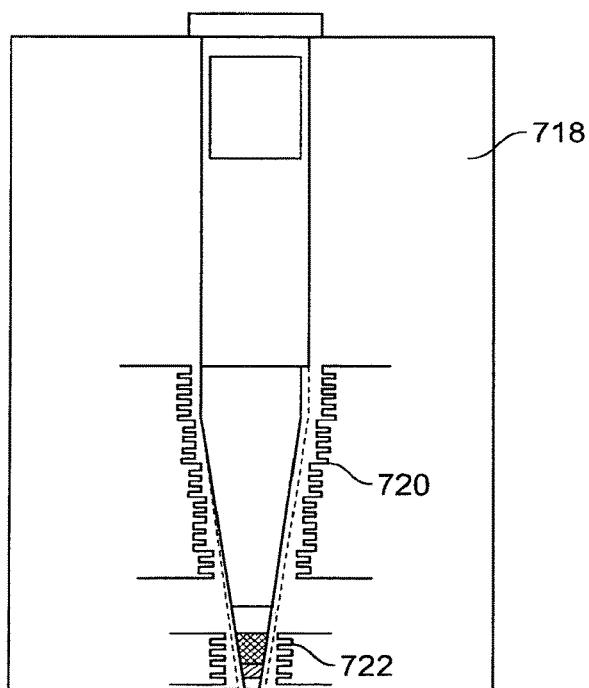


FIG. 19

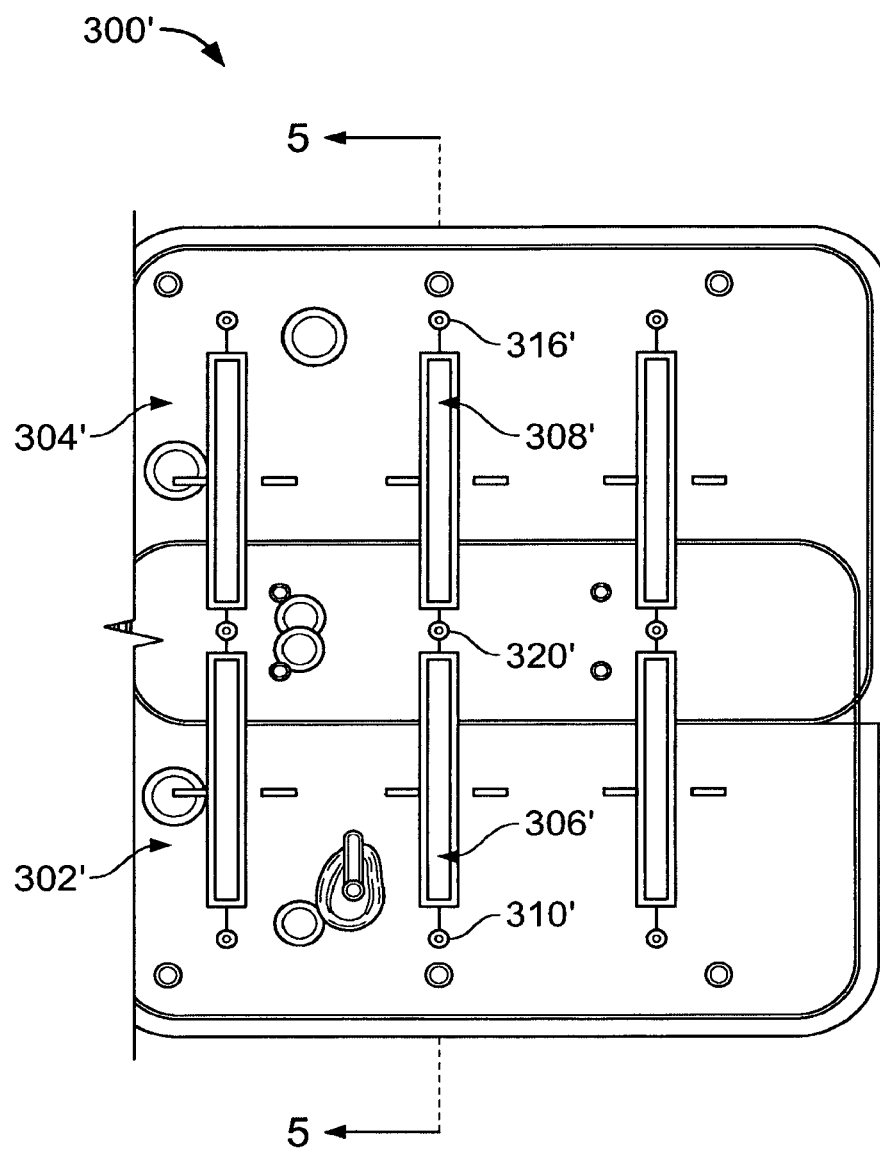


FIG. 20

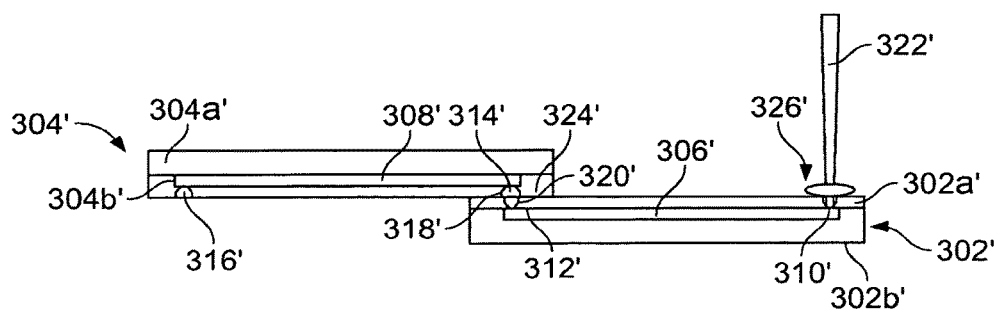


FIG. 21

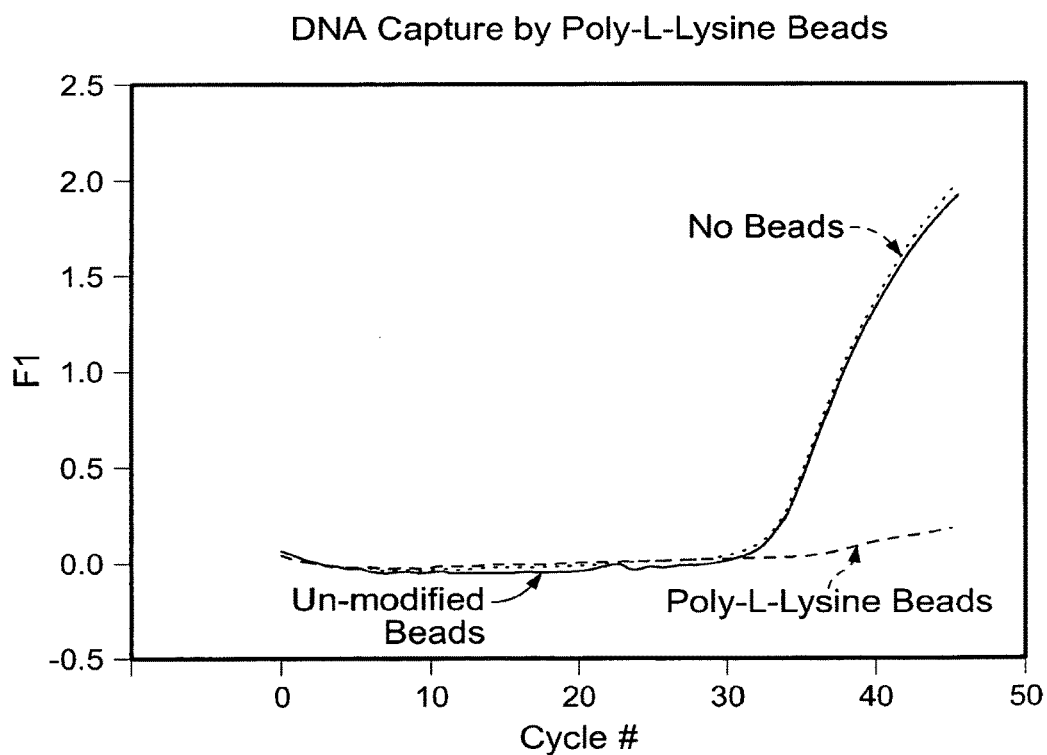


FIG. 22

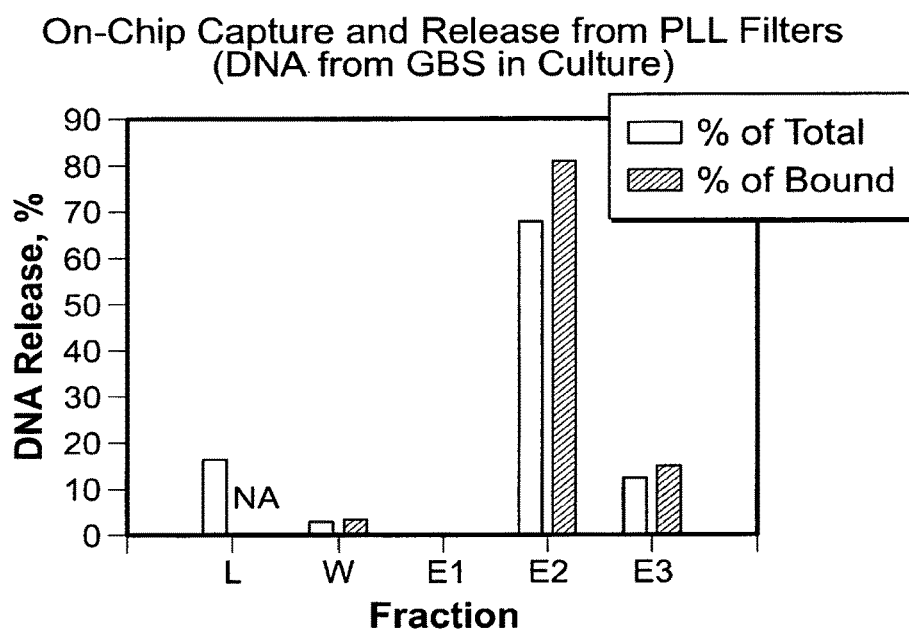


FIG. 23

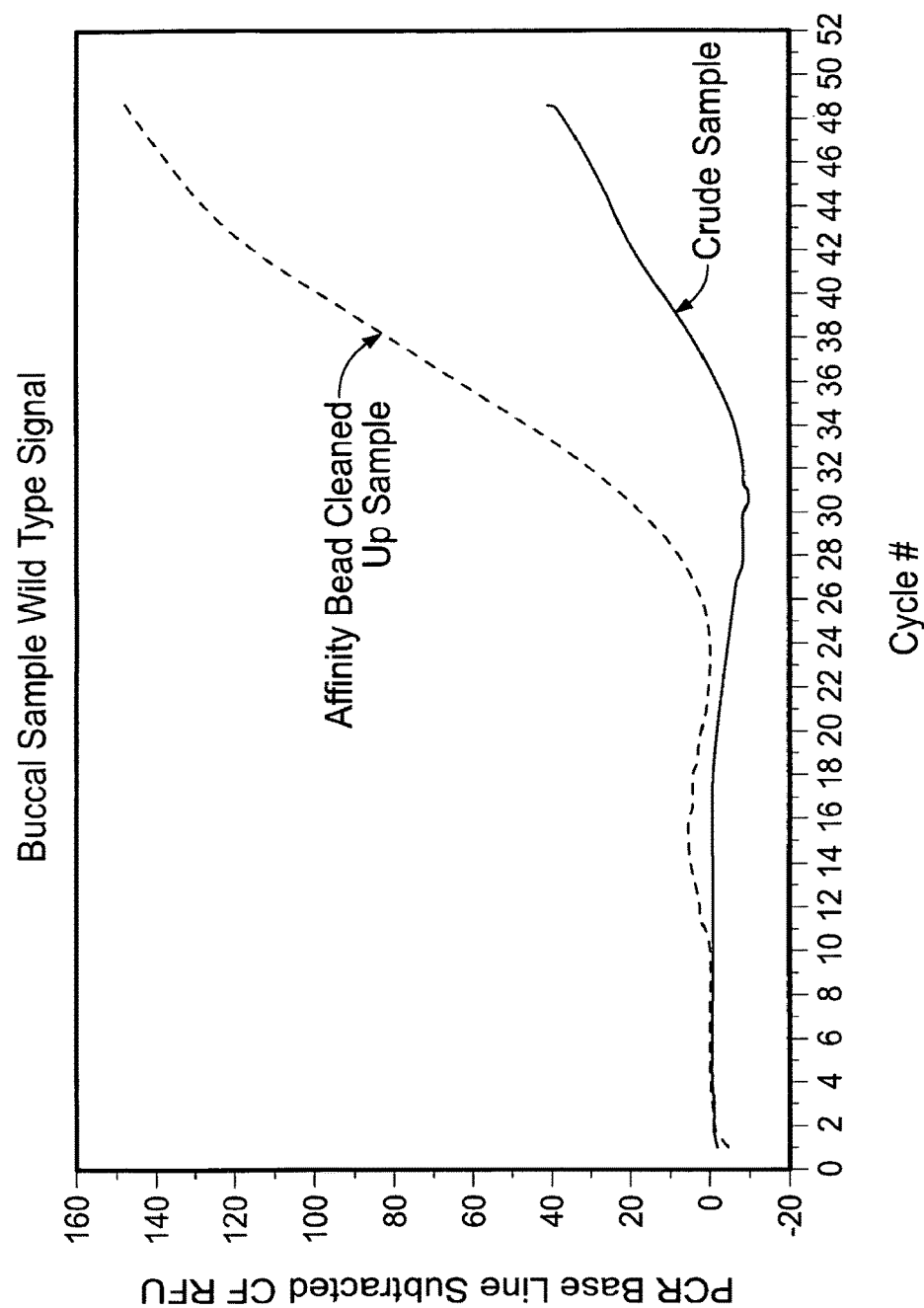


FIG. 24

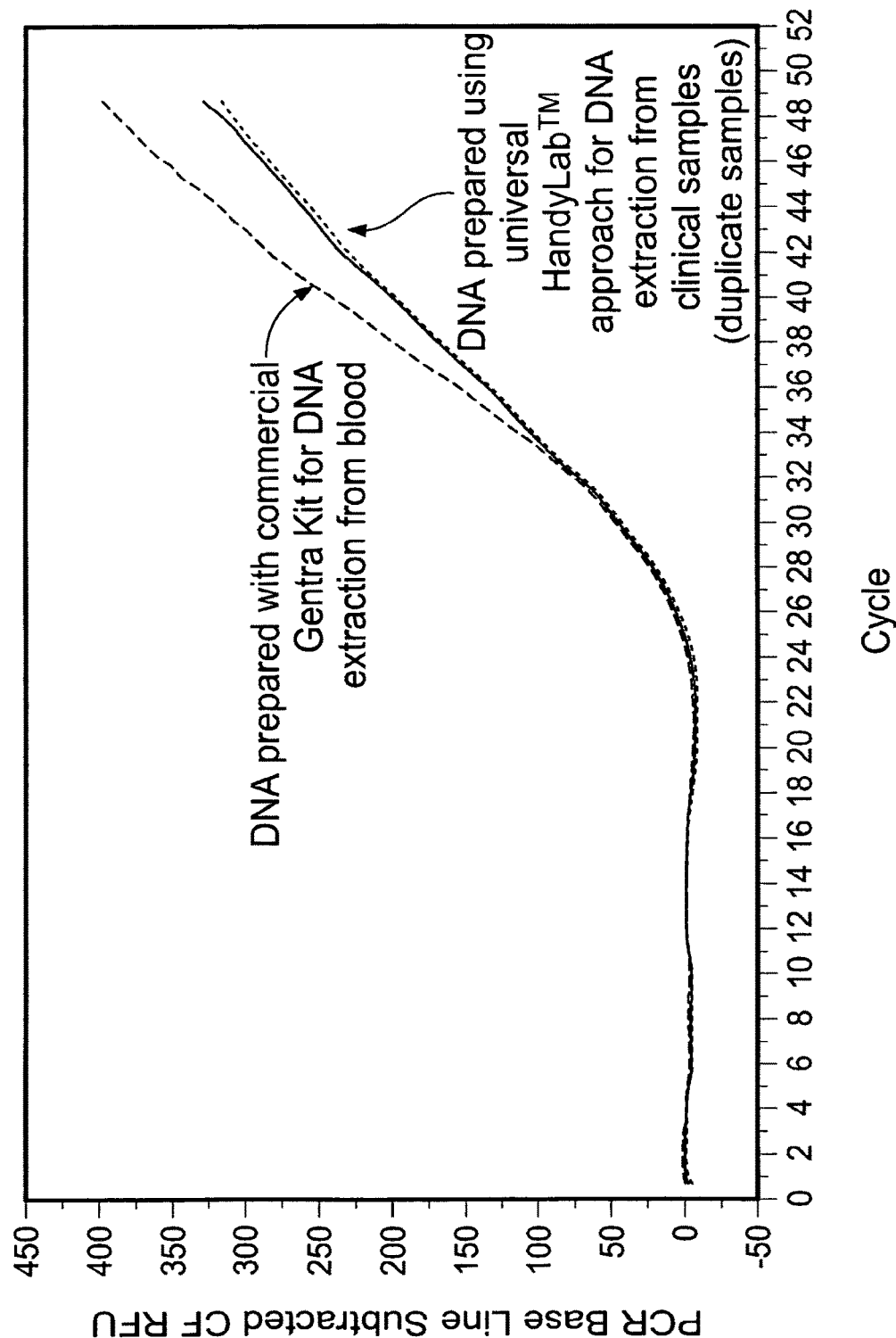


FIG. 25

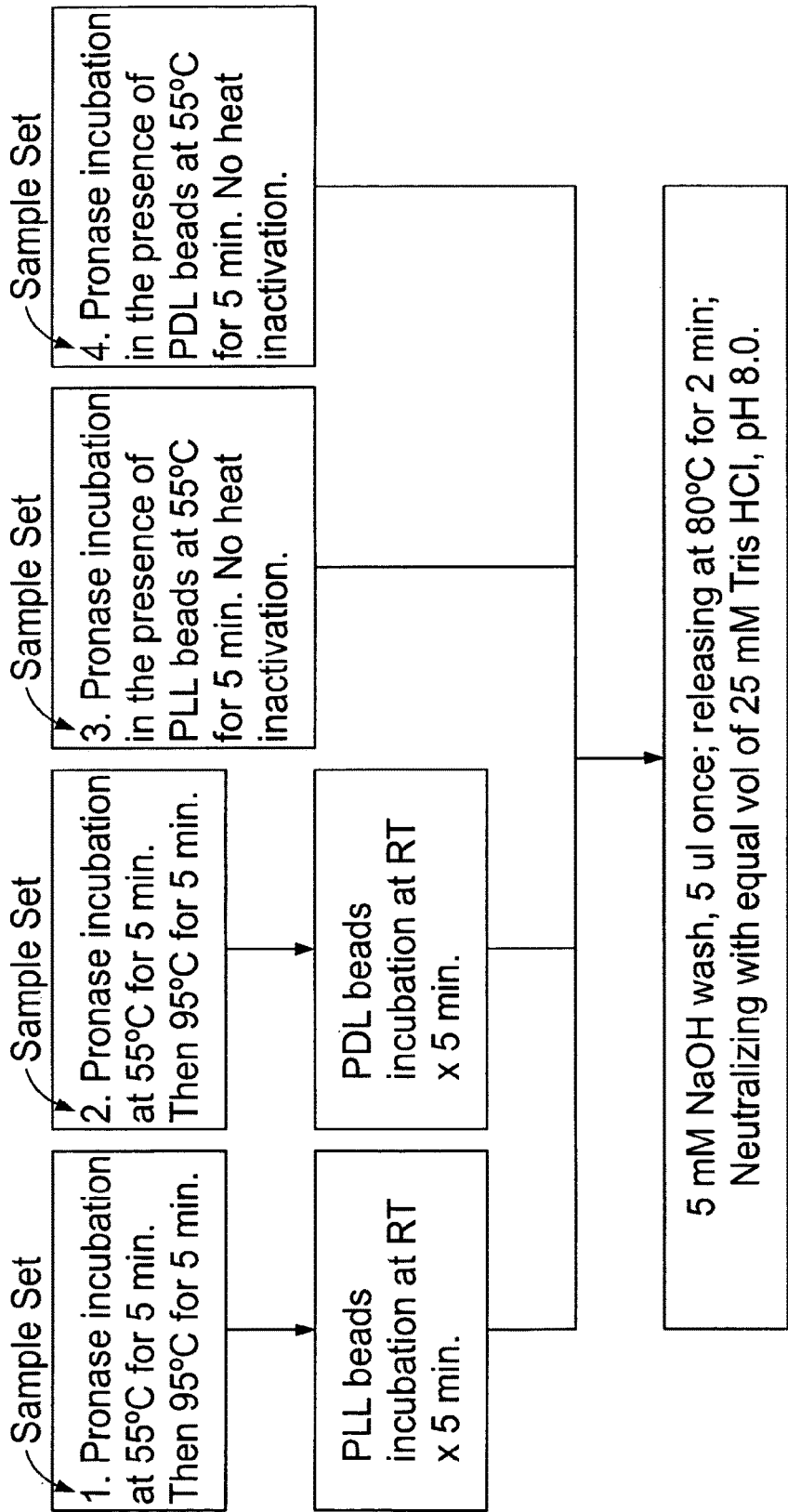


FIG. 26A

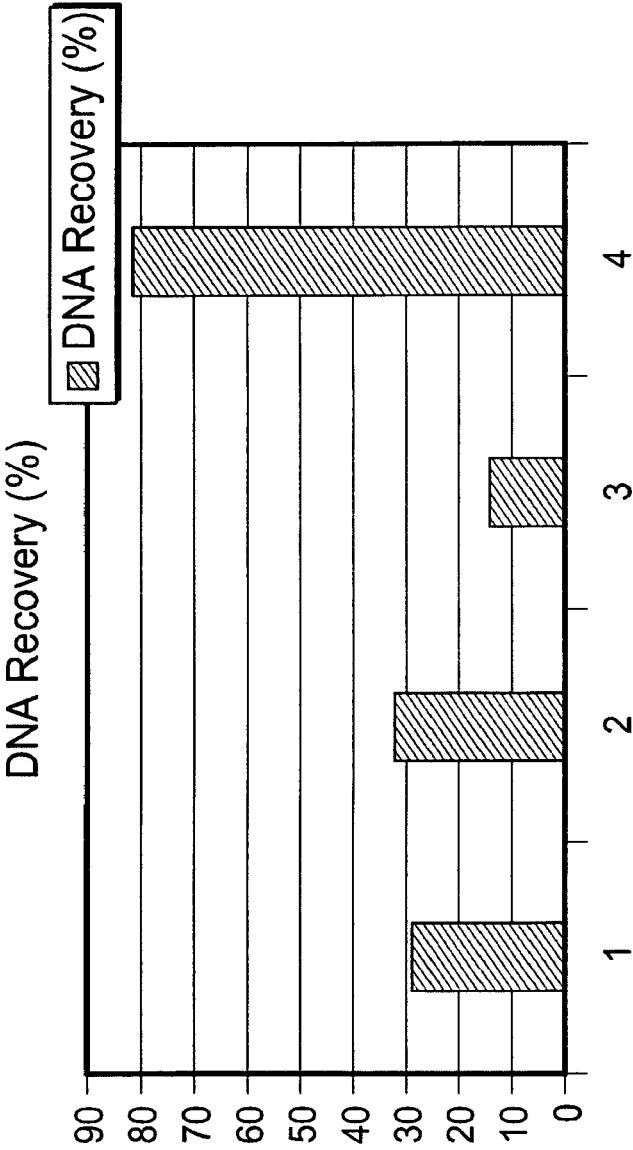


FIG. 26B

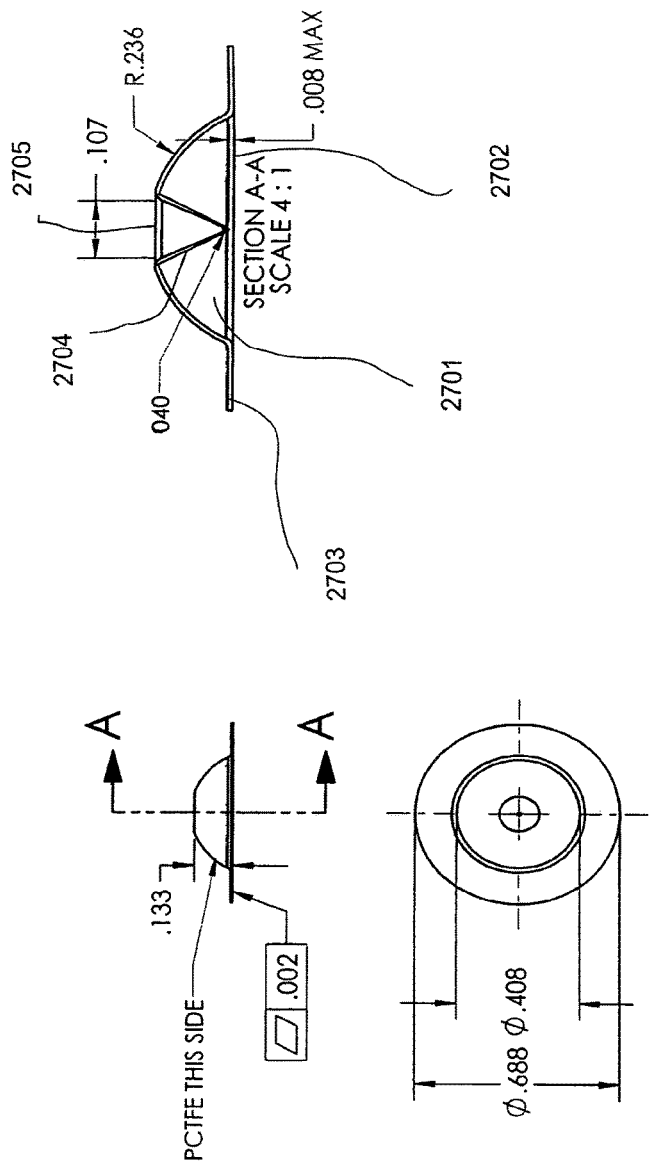


FIG. 27A

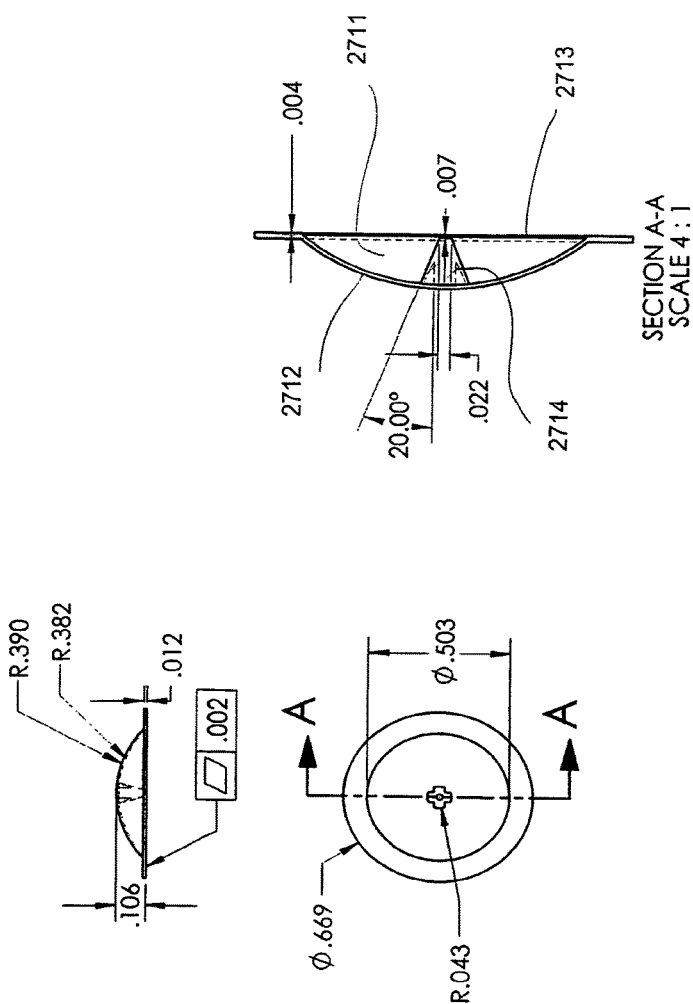


FIG. 27B

US 10,364,456 B2

1

METHOD FOR PROCESSING POLYNUCLEOTIDE-CONTAINING SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 11/281,247, filed Nov. 16, 2005 and scheduled to issue as U.S. Pat. No. 8,852,862 on Oct. 7, 2014, which is a continuation-in-part of International Application No. PCT/US2005/015345, filed May 3, 2005, which claims the benefit of priority of U.S. Provisional Application No. 60/567,174, filed May 3, 2004, and U.S. Provisional Application No. 60/645,784, filed Jan. 21, 2005. Each of these applications is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods for processing polynucleotide-containing samples as well as to related systems.

BACKGROUND

The analysis of a biological sample often includes detecting one or more polynucleotides present in the sample. One example of detection is qualitative detection, which relates, for example, to the determination of the presence of the polynucleotide and/or the determination of information related to, for example, the type, size, presence or absence of mutations, and/or the sequence of the polynucleotide. Another example of detection is quantitative detection, which relates, for example, to the determination of the amount of polynucleotide present. Detection may include both qualitative and quantitative aspects.

Detecting polynucleotides often involves the use of an enzyme. For example, some detection methods include polynucleotide amplification by polymerase chain reaction (PCR) or a related amplification technique. Other detection methods that do not amplify the polynucleotide to be detected also make use of enzymes. However, the functioning of enzymes used in such techniques may be inhibited by the presence of inhibitors present along with the polynucleotide to be detected. The inhibitors may interfere with, for example, the efficiency and/or specificity of the enzymes.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a method and related systems for processing one or more polynucleotides (e.g., to concentrate the polynucleotide(s) and/or to separate the polynucleotide(s) from inhibitor compounds (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucosal compounds, DNA binding proteins, or a saccharide) that might inhibit detection and/or amplification of the polynucleotides).

In some embodiments, the method includes contacting the polynucleotides and a relatively immobilized compound that preferentially associates with (e.g., retains) the polynucleotides as opposed to inhibitors. An exemplary compound is a poly-cationic polyamide (e.g., poly-L-lysine and/or poly-D-lysine), or polyethyleneimine (PEI), which may be bound to a surface (e.g., a surface of one or more particles). The compound retains the polynucleotides so that the polynucleotides and inhibitors may be separated, such as by washing the surface with the compound and associated polynucle-

2

otides. Upon separation, the association between the polynucleotide and compound may be disrupted to release (e.g., separate) the polynucleotides from the compound and surface.

In some embodiments, the surface (e.g., a surface of one or more particles) is modified with a poly-cationic substance such as a polyamide or PEI, which may be covalently bound to the surface. The poly-cationic polyamide may include at least one of poly-L-lysine and poly-D-lysine. In some embodiments, the poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) have an average molecular weight of at least about 7500 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have an average molecular weight of less than about 35,000 Da (e.g., an average molecular weight of less than about 30,000 Da (e.g., an average molecular weight of about 25,000 Da)). The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of at least about 15,000 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of less than about 25,000 Da (e.g., a median molecular weight of less than about 20,000 Da (e.g., a median molecular weight of about 20,000 Da)). If the polycationic material is PEI, its molecular weight is preferably in the range 600-800 Daltons.

Another aspect of the invention relates to a sample preparation device including a surface including a poly-cationic polyamide or PEI bound thereto and a sample introduction passage in communication with the surface for contacting the surface with a fluidic sample.

In some embodiments, the device includes a heat source configured to heat an aqueous liquid in contact with the surface to at least about 65° C.

In some embodiments, the device includes a reservoir of liquid having a pH of at least about 10 (e.g., about 10.5 or more). The device is configured to contact the surface with the liquid (e.g., by actuating a pressure source to move the liquid).

In some embodiments, the surface comprises surfaces of a plurality of particles.

In some embodiments, the poly-cationic polyamide includes poly-L-lysine and/or poly-D-lysine.

Another aspect of the invention relates to a method for processing a sample including providing a mixture including a liquid and an amount of polynucleotide, contacting a retention member with the mixture. The retention member may be configured to preferentially retain polynucleotides as compared to polymerase chain reaction inhibitors. Substantially all of the liquid in the mixture is removed from the retention member. The polynucleotides are released from the retention member. The polynucleotide may have a size of less than about 7.5 Mbp.

The liquid may be a first liquid and removing substantially all of the liquid from the retention member may include contacting the retention member with a second liquid.

Contacting the retention member with a second liquid can include actuating a thermally actuated pressure source to apply a pressure to the second liquid. Contacting the retention member with a second liquid can include opening a thermally actuated valve to place the second liquid in fluid communication with the retention member.

The second liquid may have a volume of less than about 50 microliters.

US 10,364,456 B2

3

The retention member may include a surface having a compound configured to bind polynucleotides preferentially to polymerase chain reaction inhibitors (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucousol compounds, DNA binding proteins, or a saccharide).

The surface may include a poly-lysine (e.g., poly-L-lysine and/or poly-D-lysine) or PEI.

The second liquid may include a detergent (e.g., SDS).

Releasing may include heating the retention member to a temperature of at least about 50° C. (e.g., at about 65° C.). The temperature may be insufficient to boil the liquid in the presence of the retention member during heating. The temperature may be 100° C. or less (e.g., less than 100° C., about 97° C. or less). The temperature may be maintained for less than about 10 minutes (e.g., for less than about 5 minutes, for less than about 3 minutes).

The releasing may be performed without centrifugation of the retention member.

In certain embodiments, PCR inhibitors are rapidly removed from clinical samples to create a PCR-ready sample. The method may comprise the preparation of a polynucleotide-containing sample that is substantially free of inhibitors. The samples may be prepared from, e.g., crude lysates resulting from thermal, chemical, ultrasonic, mechanical, electrostatic, and other lysing techniques. The samples may be prepared without centrifugation. The samples may be prepared using microfluidic devices or on a larger scale.

Another aspect of the invention relates to a retention member, e.g., a plurality of particles such as beads, comprising bound PEI, or poly-lysine, e.g., poly-L-lysine, and related methods and systems. The retention member preferentially binds polynucleotides, e.g., DNA, as compared to inhibitors. The retention member may be used to prepare polynucleotides samples for further processing, such as amplification by polymerase chain reaction.

In certain embodiments, more than 90% of a polynucleotide present in a sample may be bound to the retention member, released, and recovered.

In certain embodiments, a polynucleotide may be bound to the retention member, released, and recovered, in less than about 10 minutes (e.g., less than about 7.5 minutes, less than about 5 minutes, or less than about 3 minutes).

A polynucleotide may be bound to a retention member, released, and recovered without subjecting the polynucleotide, retention member, and/or inhibitors to centrifugation.

Separating the polynucleotides and inhibitors generally excludes subjecting the polynucleotides, inhibitors, processing region, and/or retention member to sedimentation (e.g., centrifugation).

Another aspect of the invention relates to a microfluidic device including a channel, a first mass of a thermally responsive substance (TRS) disposed on a first side of the channel, a second mass of a TRS disposed on a second side of the channel opposite the first side of the channel, a gas pressure source associated with the first mass of the TRS. Actuation of the gas pressure source drives the first mass of the TRS into the second mass of the TRS and obstructs the channel.

The microfluidic device can include a second gas pressure source associated with the second mass of the TRS. Actuation of the second gas pressure source drives the second mass of TRS into the first mass of TRS.

At least one (e.g., both) of the first and second masses of TRS may be a wax.

Another aspect of the invention relates to a method for obstructing a channel of a microfluidic device. A mass of a

4

TRS is heated and driven across the channel (e.g., by gas pressure) into a second mass of TRS. The second mass of TRS may also be driven (e.g., by gas pressure) toward the first mass of TRS.

Another aspect of the invention relates to an actuator for a microfluidic device. The actuator includes a channel, a chamber connected to the channel, at least one reservoir of encapsulated liquid disposed in the chamber, and a gas surrounding the reservoir within the chamber. Heating the chamber expands the reservoir of encapsulated liquid and pressurizes the gas. Typically the liquid has a boiling point of about 90° C. or less. The liquid may be a hydrocarbon having about 10 carbon atoms or fewer.

The liquid may be encapsulated by a polymer.

The actuator may include multiple reservoirs of encapsulated liquid disposed in the chamber.

The multiple reservoirs may be dispersed within a solid (e.g., a wax).

The multiple reservoirs may be disposed within a flexible enclosure (e.g., a flexible sack).

Another aspect of the invention relates to a method including pressurizing a gas within a chamber of a microfluidic to create a gas pressure sufficient to move a liquid within a channel of the microfluidic device. Pressurizing the gas typically expanding at least one reservoir of encapsulated liquid disposed within the chamber.

Expanding the at least one reservoir can include heating the chamber.

Pressurizing the gas can include expanding multiple reservoirs of encapsulated liquid.

Another aspect of the invention relates to a method for combining (e.g., mixing) first and second liquids and related devices. The device includes a mass of a temperature responsive substance (TRS) that separates first and second channels of the device. The device is configured to move a first liquid along the first channel so that a portion (e.g., a medial portion) of the first liquid is adjacent the TRS and to move a second liquid along the second channel so that a portion (e.g., a medial portion) of second liquid is adjacent the TRS. A heat source is actuated to move the TRS (e.g., by melting, dispersing, fragmenting). The medial portions of the first and second liquids typically combine without being separated by a gas interface. Typically, only a subset of the first liquid and a subset of the second liquid are combined. The liquids mix upon being moved along a mixing channel.

Another aspect of the invention relates to a lyophilized reagent particle and a method of making the particle.

In some embodiments, the lyophilized particles include multiple smaller particles each having a plurality of ligands that preferentially associate with polynucleotides as compared to PCR inhibitors. The lyophilized particles can also (or alternatively) include lysing reagents (e.g., enzymes) configured to lyse cells to release polynucleotides. The lyophilized particles can also (or alternatively) include enzymes (e.g., proteases) that degrade proteins.

Cells can be lysed by combining a solution of the cells with the lyophilized particles to reconstitute the particles. The reconstituted lysing reagents lyse the cells. The polynucleotides associate with ligands of the smaller particles. During lysis, the solution may be heated (e.g., radiatively using a lamp (e.g., a heat lamp)).

In some embodiments, lyophilized particles include reagents (e.g., primers, control plasmids, polymerase enzymes) for performing PCR.

A method for making lyophilized particles includes forming a solution of reagents of the particle and a cryoprotectant (e.g., a sugar or poly-alcohol). The solution is deposited

dropwise on a chilled hydrophobic surface (e.g., a diamond film or polytetrafluoroethylene surface), without contacting a cooling agent such as liquid nitrogen. The particles freeze and are subjected to reduced pressure (typically while still frozen) for a time sufficient to remove (e.g., sublimate) the solvent. The lyophilized particles may have a diameter of about 5 mm or less (e.g., about 2.5 mm or less, about 1.75 mm or less).

Another aspect of the invention relates to a liquid reservoir capable of holding a liquid (e.g., a solvent, a buffer, a reagent, or combination thereof). In general, the reservoir can have one or more of the following features.

The reservoir can include a wall that can be manipulated (e.g., pressed or depressed) to decrease a volume within the reservoir. For example, the reservoir can include a piercing member (e.g., a needle-like or otherwise pointed or sharp member) that ruptures another portion of the reservoir (e.g., a portion of the wall) to release liquid. The piercing member can be internal to the reservoir such that the piercing member ruptures the wall from an inner surface of the reservoir (e.g., wall) outwards.

In general, the wall resists passage of liquid or vapor therethrough. In some embodiments, the wall lacks stretchiness. The wall may be flexible. The wall may be, e.g., a metallic layer, e.g., a foil layer, a polymer, or a laminate including a combination thereof.

The wall may be formed by vacuum formation (e.g., applying a vacuum and heat to a layer of material to draw the layer against a molding surface). The molding surface may be concave such that the wall is provided with a generally convex surface.

Exemplary liquids held by the reservoir include water and aqueous solutions including one or more salts (e.g., magnesium chloride, sodium chloride, Tris buffer, or combination thereof). The reservoir can retain the liquid (e.g., without substantial evaporation thereof) for a period of time (e.g., at least 6 months or at least a year). In some embodiments, less than 10% (e.g., less than about 5%) by weight of the liquid evaporates over a year.

The piercing member may be an integral part of a wall of the reservoir. For example, the reservoir can include a wall having an internal projection, which may be in contact with liquid in the reservoir. The reservoir also includes a second wall opposite the piercing member. During actuation, the piercing member is driven through the second wall (e.g., from the inside out) to release liquid.

In some embodiments, a maximum amount of liquid retained by a reservoir is less than about 1 ml. For example, a reservoir may hold about 500 microliters or less (e.g., 300 microliters or less). Generally, a reservoir holds at least about 25 microliters (e.g., at least about 50 microliters). The reservoir can introduce within about 10% of the intended amount of liquid (e.g., 50 ± 5 μ l).

The reservoir can deliver a predetermined amount of liquid that is substantially air-free (e.g., substantially gas-free). Upon introduction of the liquid, the substantially air and/or gas free liquid produces few or no bubbles large enough to obstruct movement of the liquid within the microfluidic device. Use of a piercing member internal to the reservoir can enhance an ability of the reservoir to deliver substantially air and/or gas free liquids.

In some embodiments, the reservoir can be actuated to release liquid by pressing (e.g., by one's finger or thumb or by mechanical pressure actuation). The pressure may be applied directly to a wall of the reservoir or to a plunger having a piercing member. In embodiments, minimal pressure is required to actuate the reservoir. An automated

system can be used to actuate (e.g., press upon) a plurality of reservoirs simultaneously or in sequence.

In some embodiments, the reservoir does not include a piercing member. Instead, internal pressure generated within the reservoir ruptures a wall of the reservoir allowing liquid to enter the microfluidic device.

Upon actuating a reservoir to introduce liquid into the microfluidic device, liquid generally does not withdraw back into the reservoir. For example, upon actuation, the volume of the reservoir may decrease to some minimum but generally does not increase so as to withdraw liquid back into the reservoir. For example, the reservoir may stay collapsed upon actuation. In such embodiments, the flexible wall may be flexible but lack hysteresis or stretchiness. Alternatively or in combination, the reservoir may draw in air from a vent without withdrawing any of the liquid.

Actuation of the reservoir may include driving a piercing member through a wall of the reservoir.

The reservoir preserves the reactivity and composition of reagents therein (e.g., the chemicals within the reservoir may exhibit little or no change in reactivity over 6 months or a year).

The flexible wall of the reservoir can limit or prevent leaching of chemicals therethrough. The reservoir can be assembled independently of a microfluidic device and then secured to the microfluidic device.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of a microfluidic device.

FIG. 2 is a cross-sectional view of a processing region for retaining polynucleotides and/or separating polynucleotides from inhibitors.

FIG. 3 is a cross-sectional view of an actuator.

FIG. 4 is a perspective view of a microfluidic device.

FIG. 5 is a side cross-sectional view of the microfluidic device of FIG. 4.

FIGS. 6A and 6B, taken together, illustrate a perspective view of a microfluidic network of the microfluidic device of FIG. 4.

FIG. 7 illustrates an array of heat sources for operating components of the microfluidic device of FIG. 4.

FIGS. 8 and 9 illustrate a valve in the open and closed states respectively.

FIG. 10A-10D illustrate a mixing gate of the microfluidic network of FIGS. 6A and 6B and adjacent regions of the network.

FIGS. 11A-11C illustrate a reservoir with actuation mechanism.

FIGS. 12A-12C illustrate a reservoir with actuation mechanism.

FIG. 13 illustrates a reservoir with actuation mechanism.

FIGS. 14A-14B illustrate a reservoir with actuation mechanism.

FIGS. 15A-15B illustrate a reservoir with actuation mechanism.

FIG. 16 illustrates a reservoir with actuation mechanism.

FIG. 17 illustrates a reservoir with actuation mechanism.

FIG. 18 illustrates a device for separating polynucleotides and inhibitors.

FIG. 19 illustrates the device of FIG. 18 and a device for operation thereof.

FIG. 20 illustrates a microfluidic device.

FIG. 21 is a cross-section of the microfluidic device of FIG. 20 taken along 5.

FIG. 22 illustrates the retention of herring sperm DNA.

US 10,364,456 B2

7

FIG. 23 illustrates the retention and release of DNA from group B streptococci;

FIG. 24 illustrates the PCR response of a sample from which inhibitors had been removed and of a sample from which inhibitors had not been removed.

FIG. 25 illustrates the PCR response of a sample prepared in accord with the invention and a sample prepared using a commercial DNA extraction method.

FIG. 26A illustrates a flow chart showing steps performed during a method for separating polynucleotides and inhibitors.

FIG. 26B illustrates DNA from samples subjected to the method of FIG. 26A.

FIGS. 27A and 27B show, respectively, two embodiments of a reservoir with a piercing member.

DETAILED DESCRIPTION OF THE INVENTION

Analysis of biological samples often includes determining whether one or more polynucleotides (e.g., a DNA, RNA, mRNA, or rRNA) is present in the sample. For example, one may analyze a sample to determine whether a polynucleotide indicative of the presence of a particular pathogen is present. Typically, biological samples are complex mixtures. For example, a sample may be provided as a blood sample, a tissue sample (e.g., a swab of, for example, nasal, buccal, anal, or vaginal tissue), a biopsy aspirate, a lysate, as fungi, or as bacteria. Polynucleotides to be determined may be contained within particles (e.g., cells (e.g., white blood cells and/or red blood cells), tissue fragments, bacteria (e.g., gram positive bacteria and/or gram negative bacteria), fungi, spores). One or more liquids (e.g., water, a buffer, blood, blood plasma, saliva, urine, spinal fluid, or organic solvent) is typically part of the sample and/or is added to the sample during a processing step.

Methods for analyzing biological samples include providing a biological sample (e.g., a swab), releasing polynucleotides from particles (e.g., bacteria) of the sample, amplifying one or more of the released polynucleotides (e.g., by polymerase chain reaction (PCR)), and determining the presence (or absence) of the amplified polynucleotide(s) (e.g., by fluorescence detection). Biological samples, however, typically include inhibitors (e.g., mucosal compounds, hemoglobin, faecal compounds, and DNA binding proteins) that can inhibit determining the presence of polynucleotides in the sample. For example, such inhibitors can reduce the amplification efficiency of polynucleotides by PCR and other enzymatic techniques for determining the presence of polynucleotides. If the concentration of inhibitors is not reduced relative to the polynucleotides to be determined, the analysis can produce false negative results.

We describe methods and related systems for processing biological samples (e.g., samples having one or more polynucleotides to be determined). Typically, the methods and systems reduce the concentration of inhibitors relative to the concentration of polynucleotides to be determined.

Referring to FIG. 1, a microfluidic device 200 includes first, second, and third layers 205, 207, and 209 that define a microfluidic network 201 having various components configured to process a sample including one or more polynucleotides to be determined. Device 200 typically processes the sample by increasing the concentration of a polynucleotide to be determined and/or by reducing the concentration of inhibitors relative to the concentration of polynucleotide to be determined.

8

We now discuss the arrangement of components of network 201.

Network 201 includes an inlet 202 by which sample material can be introduced to the network and an output 236 by which a processed sample can be removed (e.g., expelled by or extracted from) network 201. A channel 204 extends between inlet 202 and a junction 255. A valve 206 is positioned along channel 204. A reservoir channel 240 extends between junction 255 and an actuator 244. Gates 242 and 246 are positioned along channel 240. A channel 257 extends between junction 255 and a junction 259. A valve 208 is positioned along channel 257. A reservoir channel 246 extends between junction 259 and an actuator 248. Gates 250 and 252 are positioned along channel 246. A channel 261 extends between junction 259 and a junction 263. A valve 210 and a hydrophobic vent 212 are positioned along channel 261. A channel 256 extends between junction 263 and an actuator 254. A gate 258 is positioned along channel 256.

A channel 214 extends between junction 263 and a processing chamber 220, which has an inlet 265 and an outlet 267. A channel 228 extends between processing chamber outlet 267 and a waste reservoir 232. A valve 234 is positioned along channel 228. A channel 230 extends between processing chamber outlet 267 and output 236.

We turn now to particular components of network 201.

Referring also to FIG. 2, processing chamber 220 includes a plurality of particles (e.g., beads, microspheres) 218 configured to retain polynucleotides of the sample under a first set of conditions (e.g., a first temperature and/or first pH) and to release the polynucleotides under a second set of conditions (e.g., a second, higher temperature and/or a second, more basic pH). Typically, the polynucleotides are retained preferentially as compared to inhibitors that may be present in the sample. Particles 218 are configured as a retention member 216 (e.g., a column) through which sample material (e.g., polynucleotides) must pass when moving between the inlet 265 and outlet 267 of processing region 220.

A filter 219 prevents particles 218 from passing downstream of processing region 220. A channel 287 connects filter 219 with outlet 267. Filter 219 has a surface area within processing region 220 that is larger than the cross-sectional area of inlet 265. For example, in some embodiments, the ratio of the surface area of filter 219 within processing region 220 to the cross-sectional area of inlet 265 (which cross-sectional area is typically about the same as the cross-sectional area of channel 214) is at least about 5 (e.g., at least about 10, at least about 20, at least about 30). In some embodiments, the surface area of filter 219 within processing region 220 is at least about 1 mm² (e.g., at least about 2 mm², at least about 3 mm²). In some embodiments, the cross-sectional area of inlet 265 and/or channel 214 is about 0.25 mm² or less (e.g., about 0.2 mm² or less, about 0.15 mm² or less, about 0.1 mm² or less). The larger surface area presented by filter 219 to material flowing through processing region 220 helps prevent clogging of the processing region while avoiding significant increases in the void volume (discussed below) of the processing region.

Particles 218 are modified with at least one ligand that retains polynucleotides (e.g., preferentially as compared to inhibitors). Typically, the ligands retain polynucleotides from liquids having a pH about 9.5 or less (e.g., about 9.0 or less, about 8.75 or less, about 8.5 or less). As a sample solution moves through processing region 220, polynucleotides are retained while the liquid and other solution components (e.g., inhibitors) are less retained (e.g., not

retained) and exit the processing region. In general, the ligands release polynucleotides when the pH is about 10 or greater (e.g., about 10.5 or greater, about 11.0 or greater, about 11.4 or greater). Consequently, polynucleotides can be released from the ligand modified particles into the surrounding liquid.

Exemplary ligands include, for example, polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine) and PEI. Other ligands include, for example, intercalators, poly-intercalators, minor groove binders polyamines (e.g., spermidine), homopolymers and copolymers comprising a plurality of amino acids, and combinations thereof. In some embodiments, the ligands have an average molecular weight of at least about 5000 Da (e.g., at least about 7500 Da, of at least about 15000 Da). In some embodiments, the ligands have an average molecular weight of about 50000 Da or less (e.g., about 35000, or less, about 27500 Da or less). In some embodiments, the ligand is a poly-lysine ligand attached to the particle surface by an amide bond.

In certain embodiments, the ligands are resistant to enzymatic degradation, such as degradation by protease enzymes (e.g., mixtures of endo- and exo-proteases such as pronase) that cleave peptide bonds. Exemplary protease resistant ligands include, for example, poly-D-lysine and other ligands that are enantiomers of ligands susceptible to enzymatic attack.

Particles **218** are typically formed of a material to which the ligands can be associated. Exemplary materials from which particles **218** can be formed include polymeric materials that can be modified to attach a ligand. Typical polymeric materials provide or can be modified to provide carboxylic groups and/or amino groups available to attach ligands. Exemplary polymeric materials include, for example, polystyrene, latex polymers (e.g., polycarboxylate coated latex), polyacrylamide, polyethylene oxide, and derivatives thereof. Polymeric materials that can be used to form particles **218** are described in U.S. Pat. No. 6,235,313 to Mathiowitz et al., which patent is incorporated herein by reference. Other materials include glass, silica, agarose, and amino-propyl-tri-ethoxy-silane (APES) modified materials.

Exemplary particles that can be modified with suitable ligands include carboxylate particles (e.g., carboxylate modified magnetic beads (Sera-Mag Magnetic Carboxylate modified beads, Part #3008050250, Seradyn) and Polybead carboxylate modified microspheres available from Polyscience, catalog no. 09850). In some embodiments, the ligands include poly-D-lysine and the beads comprise a polymer (e.g., polycarboxylate coated latex). In other embodiments, the ligands include PEI.

In general, the ratio of mass of particles to the mass of polynucleotides retained by the particles is no more than about 25 or more (e.g., no more than about 20, no more than about 10). For example, in some embodiments, about 1 gram of particles retains about 100 milligrams of polynucleotides.

Typically, the total volume of processing region **220** (including particles **218**) between inlet **265** and filter **219** is about 15 microliters or less (e.g., about 10 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. In some embodiments, particles **218** occupy at least about 10 percent (e.g., at least about 15 percent) of the total volume of processing region **220**. In some embodiments, particles **218** occupy about 75 percent or less (e.g., about 50 percent or less, about 35 percent or less) of the total volume of processing chamber **220**.

In some embodiments, the volume of processing region **220** that is free to be occupied by liquid (e.g., the void volume of processing region **220** including interstices between particles **218**) is about equal to the total volume minus the volume occupied by the particles. Typically, the void volume of processing region **220** is about 10 microliters or less (e.g., about 7.5 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In some embodiments, the void volume is about 50 nanoliters or more (e.g., about 100 nanoliters or more, about 250 nanoliters or more). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. For example, in an exemplary embodiment, the total volume of the processing region is about 2.3 microliters, the volume occupied by particles is about 0.3 microliters, and the volume free to be occupied by liquid (void volume) is about 2 microliters.

Particles **218** typically have an average diameter of about 20 microns or less (e.g., about 15 microns or less, about 10 microns or less). In some embodiments, particles **218** have an average diameter of at least about 4 microns (e.g., at least about 6 microns, at least about 8 microns).

In some embodiments, a volume of channel **287** between filter **219** and outlet **267** is substantially smaller than the void volume of processing region **220**. For example, in some embodiments, the volume of channel **287** between filter **219** and outlet **267** is about 35% or less (e.g., about 25% or less, about 20% or less) of the void volume. In an exemplary embodiment, the volume of channel **287** between filter **219** and outlet **267** is about 500 nanoliters.

The particle density is typically at least about 10^8 particles per milliliter (e.g., about 10^9 particles per milliliter). For example, a processing region with a total volume of about 1 microliter may include about 103 beads.

Filter **219** typically has pores with a width smaller than the diameter of particles **218**. In an exemplary embodiment, filter **219** has pores having an average width of about 8 microns and particles **218** have an average diameter of about 10 microns.

In some embodiments, at least some (e.g., all) of the particles are magnetic. In alternative embodiments, few (e.g., none) of the particles are magnetic.

In some embodiments, at least some (e.g., all) the particles are solid. In some embodiments, at least some (e.g., all) the particles are porous (e.g., the particles may have channels extending at least partially within them).

We continue discussing components of network **201**.

Channels of microfluidic network **201** typically have at least one sub-millimeter cross-sectional dimension. For example, channels of network **201** may have a width and/or a depth of about 1 mm or less (e.g., about 750 microns or less, about 500 microns, or less, about 250 microns or less).

A valve is a component that has a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Upon actuation, the valve transitions to a closed state that prevents material from passing along the channel from one side of the valve to the other. For example, valve **206** includes a mass **251** of a thermally responsive substance (TRS) that is relatively immobile at a first temperature and more mobile at a second temperature. A chamber **253** is in gaseous communication with mass **251**. Upon heating gas (e.g., air) in chamber **253** and heating mass **251** of TRS to the second temperature, gas pressure within chamber **253** moves mass **251** into channel **204** obstructing material from

US 10,364,456 B2

11

passing therealong. Other valves of network **201** have the same structure and operate in the same fashion as valve **206**.

A mass of TRS can be an essentially solid mass or an agglomeration of smaller particles that cooperate to obstruct the passage. Examples of TRS's include a eutectic alloy (e.g., a solder), wax (e.g., an olefin), polymers, plastics, and combinations thereof. The first and second temperatures are insufficiently high to damage materials, such as polymer layers of device **200**. Generally, the second temperature is less than about 90° C. and the first temperature is less than the second temperature (e.g., about 70° C. or less).

A gate is a component that has a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Upon actuation, the gate transitions to an open state in which material is permitted to pass from one side of the gate (e.g., upstream of the gate) to the other side of the gate (e.g., downstream of the gate). For example, gate **242** includes a mass **271** of TRS positioned to obstruct passage of material between junction **255** and channel **240**. Upon heating mass **271** to the second temperature, the mass changes state (e.g., by melting, by dispersing, by fragmenting, and/or dissolving) to permit passage of material between junction **255** and channel **240**.

The portion of channel **240** between gates **242** and **246** forms a fluid reservoir **279** configured to hold a liquid (e.g., water, an organic liquid, or combination thereof). During storage, gates **242** and **246** limit (e.g., prevent) evaporation of liquid within the fluid reservoir. During operation of device **200**, the liquid of reservoir **279** is typically used as a wash liquid to remove inhibitors from processing region **220** while leaving polynucleotides associated with particles **218**. Typically, the wash liquid is a solution having one or more additional components (e.g., a buffer, chelator, surfactant, a detergent, a base, an acid, or a combination thereof). Exemplary solutions include, for example, a solution of 10-50 mM Tris at pH 8.0, 0.5-2 mM EDTA, and 0.5%-2% SDS, a solution of 10-50 mM Tris at pH 8.0, 0.5 to 2 mM EDTA, and 0.5%-2% Triton X-100.

The portion of channel **246** between gates **250** and **252** form a fluid reservoir **281** configured like reservoir **279** to hold a liquid (e.g., a solution) with limited or no evaporation. During operation of device **200**, the liquid of reservoir **281** is typically used as a release liquid into which polynucleotides that had been retained by particles **218** are released. An exemplary release liquid is an hydroxide solution (e.g., a NaOH solution) having a concentration of, for example, between about 2 mM hydroxide (e.g., about 2 mM NaOH) and about 500 mM hydroxide (e.g., about 500 mM NaOH). In some embodiments, liquid in reservoir **281** is an hydroxide solution having a concentration of about 25 mM or less (e.g., an hydroxide concentration of about 15 mM).

Reservoirs **279**, **281** typically hold at least about 0.375 microliters of liquid (e.g., at least about 0.750 microliters, at least about 1.25 microliters, at least about 2.5 microliters). In some embodiments, reservoirs **279**, **281** hold about 7.5 microliters or less of liquid (e.g., about 5 microliters or less, about 4 microliters or less, about 3 microliters or less).

An actuator is a component that provides a gas pressure that can move material (e.g., sample material and/or reagent material) between one location of network **201** and another location. For example, referring to FIG. 3, actuator **244** includes a chamber **272** having a mass **273** of thermally expansive material (TEM) therein. When heated, the TEM expands decreasing the free volume within chamber **272** and pressurizing the gas (e.g., air) surrounding mass **273** within chamber **272**. Typically, gates **246** and **242** are actuated with

12

actuator **244**. Consequently, the pressurized gas drives liquid in fluid reservoir **279** towards junction **255**. In some embodiments, actuator **244** can generate a pressure differential of more than about 3 psi (e.g., at least about 4 psi, at least about 5 psi) between the actuator and junction **255**.

The TEM includes a plurality of sealed liquid reservoirs (e.g., spheres) **275** dispersed within a carrier **277**. Typically, the liquid is a high vapor pressure liquid (e.g., isobutane and/or isopentane) sealed within a casing (e.g., a polymeric casing formed of monomers such as vinylidene chloride, acrylonitrile and methylmethacrylate). Carrier **277** has properties (e.g., flexibility and/or an ability to soften (e.g., melt) at higher temperatures) that permit expansion of the reservoirs **275** without allowing the reservoirs to pass along channel **240**. In some embodiments, carrier **277** is a wax (e.g., an olefin) or a polymer with a suitable glass transition temperature. Typically, the reservoirs make up at least about 25 weight percent (e.g., at least about 35 weight percent, at least about 50 weight percent) of the TEM. In some embodiments, the reservoirs make up about 75 weight percent or less (e.g., about 65 weight percent or less, about 50 weight percent or less) of the TEM. Suitable sealed liquid reservoirs can be obtained from Expancel (Akzo Nobel).

When the TEM is heated (e.g., to a temperature of at least about 50° C. (e.g., to at least about 75° C., at least about 90° C.)), the liquid vaporizes and increases the volume of each sealed reservoir and of mass **273**. Carrier **277** softens allowing mass **273** to expand. Typically, the TEM is heated to a temperature of less than about 150° C. (e.g., about 125° C. or less, about 110° C. or less, about 100° C. or less) during actuation. In some embodiments, the volume of the TEM expands by at least about 5 times (e.g., at least about 10 times, at least about 20 times, at least about 30 times).

A hydrophobic vent (e.g., vent **212**) is a structure that permits gas to exit a channel while limiting (e.g., preventing) liquid from exiting the channel. Typically, hydrophobic vents include a layer of porous hydrophobic material (e.g., a porous filter such as a porous hydrophobic membrane from Osmonics) that defines a wall of the channel. As discussed below, hydrophobic vents can be used to position a microdroplet of sample at a desired location within network **201**.

The hydrophobic vents of the present invention are preferably constructed so that the amount of air that escapes through them is maximized while minimizing the volume of the channel below the vent surface. Accordingly, it is preferable that the vent is constructed so as to have a hydrophobic membrane of large surface area and a shallow cross section of the microchannel below the vent surface.

Hydrophobic vents typically have a length of at least about 2.5 mm (e.g., at least about 5 mm, at least about 7.5 mm) along a channel. The length of the hydrophobic vent is typically at least about 5 times (e.g., at least about 10 times, at least about 20 times) larger than a depth of the channel within the hydrophobic vent. For example, in some embodiments, the channel depth within the hydrophobic vent is about 300 microns or less (e.g., about 250 microns or less, about 200 microns or less, about 150 microns or less).

The depth of the channel within the hydrophobic vent is typically about 75% or less (e.g., about 65% or less, about 60% or less) of than the depth of the channel upstream and downstream of the hydrophobic vent. For example, in some embodiments the channel depth within the hydrophobic vent is about 150 microns and the channel depth upstream and downstream of the hydrophobic vent is about 250 microns.

A width of the channel within the hydrophobic vent is typically at least about 25% wider (e.g., at least about 50% wider) than a width of the channel upstream from the vent

US 10,364,456 B2

13

and downstream from the vent. For example, in an exemplary embodiment, the width of the channel within the hydrophobic vent is about 400 microns and the width of the channel upstream and downstream from the vent is about 250 microns.

Microfluidic device **200** can be fabricated as desired. Typically, layers **205**, **207**, and **209** are formed of a polymeric material. Components of network **201** are typically formed by molding (e.g., by injection molding) layers **207**, **209**. Layer **205** is typically a flexible polymeric material (e.g., a laminate) that is secured (e.g., adhesively and/or thermally) to layer **207** to seal components of network **201**. Layers **207** and **209** may be secured to one another using adhesive.

In use, device **200** is typically thermally associated with an array of heat sources configured to operate the components (e.g., valves, gates, actuators, and processing region **220**) of the device. In some embodiments, the heat sources are operated by an operating system, which operates the device during use. The operating system includes a processor (e.g., a computer) configured to actuate the heat sources according to a desired protocol. Processors configured to operate microfluidic devices are described in U.S. application Ser. No. 09/819,105, filed Mar. 28, 2001, which application is incorporated herein by reference. In other embodiments, the heat sources are integral with the device itself.

Device **200** may be operated as follows. Valves of network **201** are configured in the open state. Gates of network **201** are configured in the closed state. A fluidic sample comprising polynucleotides is introduced to network **201** via inlet **202**. For example, sample can be introduced with a syringe having a Luer fitting. The syringe provides pressure to initially move the sample within network **201**. Sample passes along channels **204**, **257**, **261**, and **214** to inlet **265** of processing region **220**. The sample passes through processing region **220**, exits via outlet **267**, and passes along channel **228** to waste chamber **232**. When the trailing edge (e.g., the upstream liquid-gas interface) of the sample reaches hydrophobic vent **212**, pressure provided by the introduction device (e.g., the syringe) is released from network **201** stopping further motion of the sample.

Typically, the amount of sample introduced is about 500 microliters or less (e.g., about 250 microliters or less, about 100 microliters or less, about 50 microliters or less, about 25 microliters or less, about 10 microliters or less). In some embodiments, the amount of sample is about 2 microliters or less (e.g., of about 0.5 microliters or less).

Polynucleotides entering processing region **220** pass through interstices between the particles **218**. Polynucleotides of the sample contact retention member **216** and are preferentially retained as compared to liquid of the sample and certain other sample components (e.g., inhibitors). Typically, retention member **220** retains at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **220**. Liquid of the sample and inhibitors present in the sample exit the processing region **220** via outlet **267** and enter waste chamber **232**. Processing region **220** is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during introduction of the sample.

Processing continues by washing retention member **216** with liquid of reservoir **279** to separate remaining inhibitors from polynucleotides retained by retention member **216**. To wash retention member **216**, valve **206** is closed and gates **242**, **246** of first reservoir **240** are opened. Actuator **244** is actuated and moves wash liquid within reservoir **279** along

14

channels **257**, **261**, and **214**, through processing region **220**, and into waste reservoir **232**. The wash liquid moves sample that may have remained within channels **204**, **257**, **261**, and **214** through the processing region and into waste chamber **232**. Once the trailing edge of the wash liquid reaches vent **212**, the gas pressure generated by actuator **244** is vented and further motion of the liquid is stopped.

The volume of wash liquid moved by actuator **244** through processing region **220** is typically at least about 2 times the void volume of processing region **220** (e.g., at least about 3 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less). Processing region is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during washing. Exemplary wash fluids include liquids discussed with respect to reservoirs **279** and **281**.

Processing continues by releasing polynucleotides from retention member **216**. Typically, wash liquid from reservoir **279** is replaced with release liquid (e.g., an hydroxide solution) from reservoir **281** before releasing the polynucleotides. Valve **208** is closed and gates **250**, **252** are opened. Actuator **248** is actuated thereby moving release liquid within reservoir **281** along channels **261**, **214** and into processing region **220** and in contact with retention member **216**. When the trailing edge of release liquid from reservoir **281** reaches hydrophobic vent **212**, pressure generated by actuator **248** is vented stopping the further motion of the liquid. The volume of liquid moved by actuator **248** through processing region **220** is typically at least about equal to the void volume of the processing region **220** (e.g., at least about 2 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less).

Once retention member **216** with retained polynucleotides has been contacted with liquid from reservoir **281**, a releasing step is typically performed. Typically, the releasing step includes heating release liquid present within processing region **216**. Generally, the liquid is heated to a temperature insufficient to boil liquid in the presence of the retention member. In some embodiments, the temperature is 100° C. or less (e.g., less than 100° C., about 97° C. or less). In some embodiments, the temperature is about 65° C. or more (e.g., about 75° C. or more, about 80° C. or more, about 90° C. or more). In some embodiments, the temperature maintained for about 1 minute or more (e.g., about 2 minutes or more, about 5 minutes or more, about 10 minutes or more). In some embodiments, the temperature is maintained for about 30 minutes (e.g., about 15 minutes or less, about 10 minutes or less, about 5 minutes or less). In an exemplary embodiment, processing region **220** is heated to between about 65 and 90° C. (e.g., to about 70° C.) for between about 1 and 7 minutes (e.g., for about 2 minutes).

The polynucleotides are released into the liquid present in the processing region **220** (e.g., the polynucleotides are typically released into an amount of release liquid having a volume about the same as the void volume of the processing region **220**). Typically, the polynucleotides are released into about 10 microliters or less (e.g., about 5 microliters or less, about 2.5 microliters or less) of liquid.

In certain embodiments, the ratio of the volume of original sample moved through the processing region **220** to the volume of liquid into which the polynucleotides are released is at least about 10 (e.g., at least about 50, at least about 100, at least about 250, at least about 500, at least about 1000). In some embodiments, polynucleotides from a sample having a volume of about 2 ml can be retained within the processing region, and released into about 4 microliters or

less (e.g., about 3 microliters or less, about 2 microliters or less, about 1 microliter or less) of liquid.

The liquid into which the polynucleotides are released typically includes at least about 50% (e.g., at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **220**. The concentration of polynucleotides present in the release liquid may be higher than in the original sample because the volume of release liquid is typically less than the volume of the original liquid sample moved through the processing region. For example the concentration of polynucleotides in the release liquid may be at least about 10 times greater (e.g., at least about 25 times greater, at least about 100 times greater) than the concentration of polynucleotides in the sample introduced to device **200**. The concentration of inhibitors present in the liquid into which the polynucleotides are released is generally less than concentration of inhibitors in the original fluidic sample by an amount sufficient to increase the amplification efficiency for the polynucleotides.

The time interval between introducing the polynucleotide containing sample to processing region **220** and releasing the polynucleotides into the release liquid is typically about 15 minutes or less (e.g., about 10 minutes or less, about 5 minutes or less).

Liquid including the released polynucleotides may be removed from the processing region **220** as follows. Valves **210** and **234** are closed. Gates **238** and **258** are opened. Actuator **254** is actuated to generate pressure that moves liquid and polynucleotides from processing region **220**, into channel **230**, and toward outlet **236**. The liquid with polynucleotides can be removed using, for example, a syringe or automated sampling device. Depending upon the liquid in contact with retention member **216** during polynucleotide release, the solution with released polynucleotide may be neutralized with an amount of buffer (e.g., an equal volume of 25-50 mM Tris-HCl buffer pH 8.0).

While releasing the polynucleotides has been described as including a heating step, the polynucleotides may be released without heating. For example, in some embodiments, the liquid of reservoir **281** has an ionic strength, pH, surfactant concentration, composition, or combination thereof that releases the polynucleotides from the retention member.

While the polynucleotides have been described as being released into a single volume of liquid present within processing region **220**, other configurations can be used. For example, polynucleotides may be released with the concomitant (stepwise or continuous) introduction of fluid into and/or through processing region **220**. In such embodiments, the polynucleotides may be released into liquid having a volume of about 10 times or less (e.g., about 7.5 times or less, about 5 times or less, about 2.5 times or less, about 2 times or less) than the void volume of the processing region **220**.

While reservoirs **279**, **281** have been described as holding liquids between first and second gates, other configurations can be used. For example, liquid for each reservoir may be held within a pouch (e.g., a blister pack) isolated from network **201** by a generally impermeable membrane. The pouch is configured so that a user can rupture the membrane driving liquid into reservoirs **279**, **281** where actuators **244**, **248** can move the liquid during use.

While processing regions have been described as having microliter scale dimensions, other dimensions can be used. For example, processing regions with surfaces (e.g., particles) configured to preferentially retain polynucleotides as

opposed to inhibitors may have large volumes (e.g., many tens of microliters or more, at least about 1 milliliter or more). In some embodiments, the processing region has a bench-top scale.

While processing region **220** has been described as having a retention member formed of multiple surface-modified particles, other configurations can be used. For example, in some embodiments, processing region **220** includes a retention member configured as a porous member (e.g., a filter, a porous membrane, or a gel matrix) having multiple openings (e.g., pores and/or channels) through which polynucleotides pass. Surfaces of the porous member are modified to preferentially retain polynucleotides. Filter membranes available from, for example, Osmonics, are formed of polymers that may be surface-modified and used to retain polynucleotides within processing region **220**. In some embodiments, processing region **220** includes a retention member configured as a plurality of surfaces (e.g., walls or baffles) through which a sample passes. The walls or baffles are modified to preferentially retain polynucleotides.

While processing region **220** has been described as a component of a microfluidic network, other configurations can be used. For example, in some embodiments, the retention member can be removed from a processing region for processing elsewhere. For example, the retention member may be contacted with a mixture comprising polynucleotides and inhibitors in one location and then moved to another location at which the polynucleotides are removed from the retention member.

While reservoirs **275** have been shown as dispersed within a carrier, other configurations may be used. For example, reservoirs **275** can be encased within a flexible enclosure (e.g., a membrane, for example, an enclosure such as a sack). In some embodiments, reservoirs are loose within chamber **272**. In such embodiments, actuator **244** may include a porous member having pores too small to permit passage of reservoirs **275** but large enough to permit gas to exit chamber **272**.

Microfluidic devices with various components are described in U.S. provisional application No. 60/553,553 filed Mar. 17, 2004 by Parunak et al., which application is incorporated herein by reference.

While microfluidic device **300** has been described as configured to receive polynucleotides already released from cells, microfluidic devices can be configured to release polynucleotides from cells (e.g., by lysing the cells). For example, referring to FIGS. **4**, **5**, **6A**, and **6B**, a microfluidic device **300** includes a sample lysing chamber **302** in which cells are lysed to release polynucleotides therein. Microfluidic device **300** further includes substrate layers **L1-L3**, a microfluidic network **304** (only portions of which are seen in FIG. **4**), and liquid reagent reservoirs **R1-R4**. Liquid reagent reservoirs **R1-R4** hold liquid reagents (e.g., for processing sample material) and are connected to network **304** by reagent ports **RP1-RP4**.

Network **304** is substantially defined between layers **L2** and **L3** but extends in part between all three layers **L1-L3**. Microfluidic network **304** includes multiple components including channels **Ci**, valves **Vi**, double valves **V'i**, gates **Gi**, mixing gates **MGi**, vents **Hi**, gas actuators (e.g., pumps) **Pi**, a first processing region **B1**, a second processing region **B2**, detection zones **Di**, air vents **AVi**, and waste zones **Wi**.

Components of network **304** are typically thermally actuated. As seen in FIG. **7**, a heat source network **312** includes heat sources (e.g., resistive heat sources) having locations that correspond to components of microfluidic network **304**. For example, the locations of heat sources **HPi** correspond

US 10,364,456 B2

17

to the locations of actuators P_i , the locations of heat sources HG_i correspond to locations of gates G_i and mixing gates MG_i , the locations of heat sources HV_i correspond to the locations of valves V_i and double valves V'_i , and the locations of heat sources HD_i correspond to the locations of processing chambers D_i of network **304**. In use, the components of device **300** are disposed in thermal contact with corresponding heat sources of network **312**, which is typically operated using a processor as described above for device **200**. Heat source network **312** can be integral with or separate from device **300** as described for device **200**.

We next discuss components of microfluidic device **300**.

Air vents AV_i are components that allow gas (e.g., air) displaced by the movement of liquids within network **304** to be vented so that pressure buildup does not inhibit desired movement of the liquids. For example, air vent AV_2 permits liquid to move along channel C_{14} and into channel C_{16} by venting gas downstream of the liquid through vent AV_2 .

Valves V_i are components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). The valves V_i can have the same structure as valves of microfluidic device **200**.

As seen in FIGS. **8** and **9**, double valves V'_i are also components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Taking double valve V'_{11} of FIGS. **8** and **9** as an example, double valves V'_i include first and second masses **314**, **316** of a TRS (e.g., a eutectic alloy or wax) spaced apart from one another on either side of a channel (e.g., channel C_{14}). Typically, the TRS masses **314**, **316** are offset from one another (e.g., by a distance of about 50% of a width of the TRS masses or less). Material moving through the open valve passes between the first and second TRS masses **314**, **316**. Each TRS mass **314**, **316** is associated with a respective chamber **318**, **320**, which typically includes a gas (e.g., air).

The TRS masses **314**, **316** and chambers **318**, **320** of double valve V'_i are in thermal contact with a corresponding heat source HV'_{11} of heat source network **312**. Actuating heat source HV'_{11} causes TRS masses **314**, **316** to transition to a more mobile second state (e.g., a partially melted state) and increases the pressure of gas within chambers **318**, **320**. The gas pressure drives TRS masses **314**, **316** across channel C_{11} and closes valve HV'_{11} (FIG. **9**). Typically, masses **314**, **316** at least partially combine to form a mass **322** that obstructs channel C_{11} .

Returning to FIGS. **6A**, **6B**, gates G_i are components that have a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Gates G_i can have the same structure as described for gates of device **200**.

As seen in FIG. **10A-10D**, mixing gates MG_i are components that allow two volumes of liquid to be combined (e.g., mixed) within network **304**. Mixing gates MG_i are discussed further below.

Actuators P_i are components that provide a gas pressure to move material (e.g., sample material and/or reagent material) between one location of network **304** and another location. Actuators P_i can be the same as actuators of device **200**. For example, each actuator P_i includes a chamber with a mass **273** of TEM that can be heated to pressurize gas within the chamber. Each actuator P_i includes a corresponding gate G_i (e.g., gate G_2 of actuator P_1) that prevents liquid

18

from entering the chamber of the actuator. The gate is typically actuated (e.g., opened) to allow pressure created in the chamber of the actuator to enter the microfluidic network.

Waste chambers W_i are components that can receive waste (e.g., overflow) liquid resulting from the manipulation (e.g., movement and/or mixing) of liquids within network **304**. Typically, each waste chamber W_i has an associated air vent that allows gas displaced by liquid entering the chamber to be vented.

First processing region B_1 is a component that allows polynucleotides to be concentrated and/or separated from inhibitors of a sample. Processing region B_1 can be configured and operated as processing region **220** of device **200**. In some embodiments, first processing region B_1 includes a retention member (e.g., multiple particles (e.g., microspheres or beads), a porous member, multiple walls) having at least one surface modified with one or more ligands as described for processing region **220**. For example, the ligand can include one or more polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine), or polyethyleneimine. In some embodiments, particles of the retention member are disposed in lysing chamber **302** and are moved into processing region B_1 along with sample material.

Second processing region B_2 is a component that allows material (e.g., sample material) to be combined with compounds (e.g., reagents) for determining the presence of one or more polynucleotides. In some embodiments, the compounds include one or more PCR reagents (e.g., primers, control plasmids, and polymerase enzymes). Typically, the compounds are stored within processing region as one or more lyophilized particles (e.g., pellets). The particles generally have a room temperature (e.g., about 20° C.) shelf-life of at least about 6 months (e.g., at least about 12 months). Liquid entering the second processing region B_2 dissolves (e.g., reconstitutes) the lyophilized compounds.

Typically, the lyophilized particle(s) of processing region B_2 have an average volume of about 5 microliters or less (e.g., about 4 microliters or less, about 3 microliters or less, about 2 microliters or less). In some embodiments, the lyophilized particle(s) of processing region B_2 have an average diameter of about 4 mm or less (e.g., about 3 mm or less, about 2 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 2 microliters and an average diameter of about 1.35 mm.

Lyophilized particles for determining the presence of one or more polynucleotides typically include multiple compounds. In some embodiments, the lyophilized particles include one or more compounds used in a reaction for determining the presence of a polynucleotide and/or for increasing the concentration of the polynucleotide. For example, lyophilized particles can include one or more enzymes for amplifying the polynucleotide as by PCR.

We next discuss exemplary lyophilized particles that include exemplary reagents for the amplification of polynucleotides associated with group B streptococcus (GBS) bacteria. In some embodiments, the lyophilized particles include one or more of a cryoprotectant, one or more salts, one or more primers (e.g., GBS Primer F and/or GBS Primer R), one or more probes (e.g., GBS Probe—FAM), one or more internal control plasmids, one or more specificity controls (e.g., *Streptococcus pneumoniae* DNA as a control for PCR of GBS), one or more PCR reagents (e.g., dNTPs and/or dUTPs), one or more blocking or bulking agents (e.g., non-specific proteins (e.g., bovine serum albumin (BSA), RNaseA, or gelatin), and a polymerase (e.g., gly-

US 10,364,456 B2

19

erol-free Taq Polymerase). Of course, other components (e.g., other primers and/or specificity controls) can be used for amplification of other polynucleotides.

Cryoprotectants generally help increase the stability of the lyophilized particles and help prevent damage to other compounds of the particles (e.g., by preventing denaturation of enzymes during preparation and/or storage of the particles). In some embodiments, the cryoprotectant includes one or more sugars (e.g., one or more disaccharides (e.g., trehalose, melezitose, raffinose)) and/or one or more polyalcohols (e.g., mannitol, sorbitol).

Lyophilized particles can be prepared as desired. Typically, compounds of the lyophilized particles are combined with a solvent (e.g., water) to make a solution, which is then placed (e.g., in discrete aliquots (e.g., drops) such as by pipette) onto a chilled hydrophobic surface (e.g., a diamond film or a polytetrafluorethylene surface). In general, the temperature of the surface is reduced to near the temperature of liquid nitrogen (e.g., about -150°F . or less, about -200°F . or less, about -275°F . or less), such as by use of a cooling bath of a cryogenic agent directly underneath. It is to be noted that the solution is dispensed without contacting the cryogenic agent. The solution freezes as discrete particles. The frozen particles are subjected to a vacuum while still frozen for a pressure and time sufficient to remove the solvent (e.g., by sublimation) from the pellets.

In general, the concentrations of the compounds in the solution from which the particles are made is higher than when reconstituted in the microfluidic device. Typically, the ratio of the solution concentration to the reconstituted concentration is at least about 3 (e.g., at least about 4.5). In some embodiments, the ratio is about 6.

An exemplary solution for preparing lyophilized pellets for use in the amplification of polynucleotides indicative of the presence of GBS can be made by combining a cryoprotectant (e.g., 120 mg of trehalose as dry powder), a buffer solution (e.g., 48 microliters of a solution of 1M Tris at pH 8.4, 2.5M KCl, and 200 mM MgCl_2), a first primer (e.g., 1.92 microliters of 500 micromolar GBS Primer F (Invitrogen)), a second primer (e.g., 1.92 microliters of 500 micromolar GBS Primer R (Invitrogen)), a probe (e.g., 1.92 microliters of 250 micromolar GBS Probe—FAM (IDT/Biosearch Technologies)), an control probe (e.g., 1.92 microliters of 250 micromolar Cal Orange 560 (Biosearch Technologies)), a template plasmid (e.g., 0.6 microliters of a solution of 105 copies plasmid per microliter), a specificity control (e.g., 1.2 microliters of a solution of 10 nanograms per microliter (e.g., about 5,000,000 copies per microliter) streptococcus pneumoniae DNA (ATCC)), PCR reagents (e.g., 4.8 microliters of a 100 millimolar solution of dNTPs (Epicenter) and 4 microliters of a 20 millimolar solution of dUTPs (Epicenter)), a bulking agent (e.g., 24 microliters of a 50 milligram per milliliter solution of BSA (Invitrogen)), a polymerase (e.g., 60 microliters of a 5 U per microliter solution of glycerol-free Taq Polymerase (Invitrogen/Ependorf)) and a solvent (e.g., water) to make about 400 microliters of solution. About 200 aliquots of about 2 microliters each of this solution are frozen and desolvated as described above to make 200 pellets. When reconstituted, the 200 particles make a PCR reagent solution having a total volume of about 2.4 milliliters.

As seen in FIG. 5, reagent reservoirs R_i are configured to hold liquid reagents (e.g., water, buffer solution, hydroxide solution) separated from network 304 until ready for use. Reservoirs R_1 include an enclosure 329 that defines a sealed space 330 for holding liquids. Each space 330 is separated from reagent port R_{Pi} and network 304 by a lower wall 333

20

of enclosure 329. A capping material 341 (e.g., a laminate, adhesive, or polymer layer) may overlie an upper wall of the enclosure.

A portion of enclosure 329 is formed as an actuation mechanism (e.g., a piercing member 331) oriented toward the lower wall 333 of each enclosure. When device 300 is to be used, reagent reservoirs R_i are actuated by depressing piercing member 331 to puncture wall 333. Piercing member 331 can be depressed by a user (e.g., with a thumb) or by the operating system used to operate device 300.

Wall 333 is typically formed of a material having a low vapor transmission rate (e.g., Aclar, a metallized (e.g. aluminum) laminate, a plastic, or a foil laminate) that can be ruptured or pierced. Reservoir 330 holds an amount of liquid suited for device 300. For example, the reservoir may hold up to about 200 microliters. The piercing member 331 may account for a portion (e.g., up to about 25%) of that volume.

In general, reservoirs R_i can be formed and filled as desired. For example, the upper wall of the enclosure can be sealed to the lower wall 333 (e.g., by adhesive and/or thermal sealing). Liquid can be introduced into the reservoir by, for example, an opening at the lower end of the piercing member 331. After filling, the opening can be sealed (e.g., by heat sealing through the localized application of heat or by the application of a sealing material (e.g., capping material 341)).

When wall 333 is punctured, fluid from the reservoir enters network 333. For example, as seen in FIGS. 5 and 6, liquid from reservoir R_2 enters network 304 by port RP_2 and travels along a channel C_2 . Gate G_3 prevents the liquid from passing along channel C_8 . Excess liquid passes along channel C_7 and into waste chamber W_2 . When the trailing edge of liquid from reservoir R_2 passes hydrophobic vent H_2 , pressure created within the reservoir is vented stopping further motion of the liquid. Consequently, network 304 receives an aliquot of liquid reagent having a volume defined by the volume of channel C_2 between a junction J_1 and a junction J_2 . When actuator P_1 is actuated, this aliquot of reagent is moved further within network 304. Reagent reservoirs R_1 , R_3 , and R_4 are associated with corresponding channels, hydrophobic vents, and actuators.

In the configuration shown, reagent reservoir R_1 typically holds a release liquid (e.g., a hydroxide solution as discussed above for device 200) for releasing polynucleotides retained within processing region B_1 . Reagent reservoir R_2 typically holds a wash liquid (e.g., a buffer solution as discussed above for device 200) for removing un-retained compounds (e.g., inhibitors) from processing region B_1 prior to releasing the polynucleotides. Reagent reservoir R_3 typically holds a neutralization buffer (e.g., 25-50 mM Tris-HCl buffer at pH 8.0). Reagent reservoir R_4 typically holds deionized water.

Lysing chamber 302 is divided into a primary lysing chamber 306 and a waste chamber 308. Material cannot pass from one of chambers 306, 308 into the other chamber without passing through at least a portion of network 304. Primary lysing chamber 306 includes a sample input port SP_1 for introducing sample to chamber 306, a sample output port SP_2 connecting chamber 306 to network 304, and lyophilized reagent LP that interact with sample material within chamber 306 as discussed below. Input port SP_1 includes a one way valve that permits material (e.g., sample material and gas) to enter chamber 306 but limits (e.g., prevents) material from exiting chamber 308 by port SP_1 . Typically, port SP_1 includes a fitting (e.g., a Luer fitting) configured to mate with a sample input device (e.g., a syringe) to form a gas-tight seal. Primary chamber 306

US 10,364,456 B2

21

typically has a volume of about 5 milliliters or less (e.g., about 4 milliliters or less). Prior to use, primary chamber 306 is typically filled with a gas (e.g., air).

Waste chamber 308 includes a waste portion W6 by which liquid can enter chamber 308 from network 304 and a vent 310 by which gas displaced by liquid entering chamber 308 can exit.

Lyophilized reagent particles LP of lysing chamber 302 include one or more compounds (e.g., reagents) configured to release polynucleotides from cells (e.g., by lysing the cells). For example, particles LP can include one or more enzymes configured to reduce (e.g., denature) proteins (e.g., proteinases, proteases (e.g., pronase), trypsin, proteinase K, phage lytic enzymes (e.g., PlyGBS)), lysozymes (e.g., a modified lysozyme such as ReadyLyse), cell specific enzymes (e.g., mutanolysin for lysing group B streptococci)).

In some embodiments, particles LP alternatively or additionally include components for retaining polynucleotides as compared to inhibitors. For example, particles LP can include multiple particles 218 surface modified with ligands as discussed above for device 200. Particles LP can include enzymes that reduce polynucleotides that might compete with a polynucleotide to be determined for binding sites on the surface modified particles. For example, to reduce RNA that might compete with DNA to be determined, particles LP may include an enzyme such as an RNAase (e.g., RNAaseA ISC BioExpress (Amresco)).

In an exemplary embodiment, particles LP cells include a cryoprotectant, particles modified with ligands configured to retain polynucleotides as compared to inhibitors, and one or more enzymes.

Typically, particles LP have an average volume of about 35 microliters or less (e.g., about 27.5 microliters or less, about 25 microliters or less, about 20 microliters or less). In some embodiments, the particles LP have an average diameter of about 8 mm or less (e.g., about 5 mm or less, about 4 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 20 microliters and an average diameter of about 3.5 mm.

Particles LP can be prepared as desired. Typically, the particles are prepared using a cryoprotectant and chilled hydrophobic surface as described above. For example, a solution for preparing particles LP can be prepared by combining a cryoprotectant (e.g., 6 grams of trehalose), a plurality of particles modified with ligands (e.g., about 2 milliliters of a suspension of carboxylate modified particles with poly-D-lysine ligands), a protease (e.g., 400 milligrams of pronase), an RNAase (e.g., 30 milligrams of RNAaseA (activity of 120 U per milligram), an enzyme that digests peptidoglycan (e.g., ReadyLyse (e.g., 160 microliters of a 30000 U per microliter solution of ReadyLyse)), a cell specific enzyme (e.g., mutanolysin (e.g., 200 microliters of a 50 U per microliter solution of mutanolysin), and a solvent (e.g., water) to make about 20 milliliters. About 1000 aliquots of about 20 microliters each of this solution are frozen and desolvated as described above to make 1000 pellets. When reconstituted, the pellets are typically used to make a total of about 200 milliliters of solution.

In use, device 300 can be operated as follows. Valves Vi and Vi' of network 304 are configured in the open state. Gates Gi and mixing gates MG_i of network 304 are configured in the closed state. Reagent ports R1-R4 are depressed to introduce liquid reagents into network 304 as discussed above. A sample is introduced to lysing chamber 302 via port SP1 and combined with lyophilized particles LP within primary lysing chamber 306. Typically, the sample includes

22

a combination of particles (e.g., cells) and a buffer solution. For example, an exemplary sample includes about 2 parts whole blood to 3 about parts buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% SDS). Another exemplary sample includes group B streptococci and a buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% Triton X-100).

In general, the volume of sample introduced is smaller than the total volume of primary lysing chamber 306. For example, the volume of sample may be about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. A typical sample has a volume of about 3 milliliters or less (e.g., about 1.5 milliliters or less). A volume of gas (e.g., air) is generally introduced to primary chamber 306 along with the sample. Typically, the volume of gas introduced is about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. The volume of sample and gas combine to pressurize the gas already present within chamber 306. Valve 307 of port SP1 prevents gas from exiting chamber 306. Because gates G3, G4, G8, and G10 are in the closed state, the pressurized sample is prevented from entering network 304 via port SP2.

The sample dissolves particles LP in chamber 306. Reconstituted lysing reagents (e.g., ReadyLyse, mutanolysin) begin to lyse cells of the sample releasing polynucleotides. Other reagents (e.g., protease enzymes such as pronase) begin to reduce or denature inhibitors (e.g., proteins) within the sample. Polynucleotides from the sample begin to associate with (e.g., bind to) ligands of particles 218 released from particles LP. Typically, the sample within chamber 306 is heated (e.g., to at least about 50° C., to at least about 60° C.) for a period of time (e.g., for about 15 minutes or less, about 10 minutes or less, about 7 minutes or less) while lysing occurs. In some embodiments, optical energy is used at least in part to heat contents of lysing chamber 306. For example, the operating system used to operate device 300 can include a light source (e.g., a lamp primarily emitting light in the infrared) disposed in thermal and optical contact with chamber 306. Chamber 306 includes a temperature sensor TS used to monitor the temperature of the sample within chamber 306. The lamp output is increased or decreased based on the temperature determined with sensor TS.

Continuing with the operation of device 300, G2 is actuated (e.g., opened) providing a path between port SP2 of primary lysing chamber 306 and port W6 of lysing waste chamber 308. The path extends along channel C9, channel C8, through processing region B1, and channel C11. Pressure within chamber 306 drives the lysed sample material (containing lysate, polynucleotides bound to particles 218, and other sample components) along the pathway. Particles 218 (with polynucleotides) are retained within processing region B1 (e.g., by a filter) while the liquid and other components of the sample flow into waste chamber 308. After a period of time (e.g., between about 2 and about 5 minutes), the pressure in lysing chamber 306 is vented by opening gate G1 to create a second pathway between ports SP2 and W6. Double valves V1' and V8' are closed to isolate lysing chamber 302 from network 304.

Operation of device 300 continues by actuating pump P1 and opening gates G2, G3 and G9. Pump P1 drives wash liquid in channel C2 downstream of junction J1 through processing region B1 and into waste chamber W5. The wash liquid removes inhibitors and other compounds not retained by particles 218 from processing region B1. When the trailing edge of the wash liquid (e.g., the upstream interface) passes hydrophobic vent H14, the pressure from actuator P1

US 10,364,456 B2

23

vents from network **304**, stopping further motion of the liquid. Double valves **V2'** and **V9'** are closed.

Operation continues by actuating pump **P2** and opening gates **G6**, **G4** and **G8** to move release liquid from reagent reservoir **R1** into processing region **B1** and into contact with particles **218**. Air vent **AV1** vents pressure ahead of the moving release liquid. Hydrophobic vent **H6** vents pressure behind the trailing edge of the release liquid stopping further motion of the release liquid. Double valves **V6'** and **V10'** are closed.

Operation continues by heating processing region **B1** (e.g., by heating particles **218**) to release the polynucleotides from particles **218**. The particles can be heated as described above for device **200**. Typically, the release liquid includes about 15 mM hydroxide (e.g., NaOH solution) and the particles are heated to about 70° C. for about 2 minutes to release the polynucleotides from the particles **218**.

Operation continues by actuating pump **P3** and opening gates **G5** and **G10** to move release liquid from process region **B1** downstream. Air vent **AV2** vents gas pressure downstream of the release liquid allowing the liquid to move into channel **C16**. Hydrophobic vent **H8** vents pressure from upstream of the release liquid stopping further movement. Double valve **V11'** and valve **V14** are closed.

Referring to FIG. **10A-10D**, mixing gate **MG11** is used to mix a portion of release liquid including polynucleotides released from particles **218** and neutralization buffer from reagent reservoir **R3**. FIG. **10A** shows the mixing gate **MG11** region prior to depressing reagent reservoir **R3** to introduce the neutralization buffer into network **304**. FIG. **10B** shows the mixing gate **MG11** region, after the neutralization buffer has been introduced into channels **C13** and **C12**. Double valve **V13'** is closed to isolate network **304** from reagent reservoir **R3**. Double valve **V12'** is closed to isolate network **304** from waste chamber **W3**. The neutralization buffer contacts one side of a mass **324** of TRS of gate **MG11**.

FIG. **10C** shows the mixing gate **MG11** region after release liquid has been moved into channel **C16**. The dimensions of microfluidic network **304** (e.g., the channel dimensions and the position of hydrophobic vent **H8**) are configured so that the portion of release liquid positioned between junctions **J3** and **J4** of channels **C16** and **C14** corresponds approximately to the volume of liquid in contact with particles **218** during the release step. In some embodiments, the volume of liquid positioned between junctions **J3** and **J4** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J3** and **J4** is about 1.75 microliters. Typically, the liquid between junctions **J3** and **J4** includes at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **B1**. Valve **V14** is closed to isolate network **304** from air vent **AV2**.

Before actuating mixing gate **MG11**, the release liquid at junction **J4** and the neutralization buffer at a junction **J6** between channels **C13** and **C12** are separated only by mass **324** of TRS (e.g., the liquids are not spaced apart by a volume of gas). To combine the release liquid and neutralization buffer, pump **P4** and gates **G12**, **G13**, and **MG11** are actuated. Pump **P4** drives the volume of neutralization liquid between junctions **J5** and **J6** and the volume of release liquid between junctions **J4** and **J3** into mixing channel **C15** (FIG. **10D**). Mass **324** of TRS typically disperses and/or melts allowing the two liquids to combine. The combined liquids include a downstream interface **335** (formed by junction **J3**)

24

and an upstream interface (formed by junction **J5**). The presence of these interfaces allows more efficient mixing (e.g., recirculation of the combined liquid) than if the interfaces were not present. As seen in FIG. **10D**, mixing typically begins near the interface between the two liquids. Mixing channel **C15** is typically at least about as long (e.g., at least about twice as long) as a total length of the combined liquids within the channel.

The volume of neutralization buffer combined with the release liquid is determined by the channel dimensions between junction **J5** and **J6**. Typically, the volume of combined neutralization liquid is about the same as the volume of combined release liquid. In some embodiments, the volume of liquid positioned between junctions **J5** and **J6** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J5** and **J6** is about 2.25 microliters (e.g., the total volume of release liquid and neutralization buffer is about 4 microliters).

Returning to FIGS. **6A**, **6B**, the combined release liquid and neutralization buffer move along mixing channel **C15** and into channel **C32** (vented downstream by air vent **AV8**). Motion continues until the upstream interface of the combined liquids passes hydrophobic vent **H11**, which vents pressure from actuator **P4** stopping further motion of the combined liquids.

Continuing with operation of device **300**, actuator **P5** and gates **G14**, **G15** and **G17** are actuated to dissolve the lyophilized PCR particle present in second processing region **B2** in water from reagent reservoir **R4**. Hydrophobic vent **H10** vents pressure from actuator **P5** upstream of the water stopping further motion. Dissolution of a PCR-reagent pellet typically occurs in about 2 minutes or less (e.g., in about 1 minute or less). Valve **V17** is closed.

Continuing with operation of device **300**, actuator **P6** and gate **G16** are actuated to drive the dissolved compounds of the lyophilized particle from processing region **B2** into channel **C31**, where the dissolved reagents mix to form a homogenous dissolved lyophilized particle solution. Actuator **P6** moves the solution into channels **C35** and **C33** (vented downstream by air vent **AV5**). Hydrophobic vent **H9** vents pressure generated by actuator **P6** upstream of the solution stopping further motion. Valves **V18**, **V19**, **V20'**, and **V22'** are closed.

Continuing with operation of device **300**, actuator **P7** and gates **G18**, **MG20** and **G22** are actuated to combine (e.g., mix) a portion of neutralized release liquid in channel **32** between gate **MG20** and gate **G22** and a portion of the dissolved lyophilized particle solution in channel **C35** between gate **G18** and **MG20**. The combined liquids travel along a mixing channel **C37** and into detection region **D2**. An air vent **AV3** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H13**, the pressure from actuator **P7** is vented and the combined liquids are positioned within detection region **D2**.

Actuator **P8** and gates **MG2**, **G23**, and **G19** are actuated to combine a portion of water from reagent reservoir **R4** between **MG2** and gate **G23** with a second portion of the dissolved lyophilized particle solution in channel **C33** between gate **G19** and **MG2**. The combined liquids travel along a mixing channel **C41** and into detection region **D1**. An air vent **AV4** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H12**, the pressure from actuator **P8** is vented and the combined liquids are positioned within detection region **D1**.

Continuing with operation of device **300**, double valves **V26'** and **V27'** are closed to isolate detection region **D1** from network **304** and double valves **V24'** and **V25'** are closed to isolate detection region **D2** from network **304**. The contents of each detection region (neutralized release liquid with sample polynucleotides in detection region **D2** with PCR reagents from dissolved lyophilized particle solution and deionized water with PCR reagents from dissolved lyophilized particle solution in detection region **D1**) are subjected to heating and cooling steps to amplify polynucleotides (if present in detection region **D2**). The double valves of each detection region prevent evaporation of the detection region contents during heating. The amplified polynucleotides are typically detected using fluorescence detection.

While reservoirs have been shown as having a piercing member formed of a wall of the reservoir, other configurations are possible. For example, in some embodiments, the reservoir includes a needle-like piercing member that extends through an upper wall of the reservoir into the sealed space toward a lower wall of the reservoir. The upper wall of the reservoir may be sealed at the needle-like piercing member (e.g., with an adhesive, an epoxy). In use, the upper wall is depressed driving the piercing member through the lower wall forcing liquid in the sealed space to enter a microfluidic network.

While reservoirs have been described as including an actuation mechanism (e.g., a piercing member), other configurations are possible. For example, in some embodiments, a lower wall of the sealed space of the reservoir includes a weakened portion that overlies an opening to a microfluidic network. The lower wall material (e.g., laminate, polymer film, or foil) that overlies the opening is thick enough to prevent loss of the liquid within the sealed space but thin enough to rupture upon the application of pressure to the liquid therein. Typically, the material overlying the opening is thinner than the adjacent material. Alternatively, or in addition, the weakened material can be formed by leaving this material relatively unsupported as compared to the surrounding material of the lower wall.

While reservoirs have been described as having a sealed space formed in part by a wall of the sealed space, other configurations are possible. For example, referring to FIG. **11A**, a reservoir includes a plunger-like actuation mechanism (e.g., a piercing member **342**) and a gasket-like sealed space **343** having upper and lower layers **344**, **345** respectively (e.g., upper and lower laminate layers). Liquid is sealed between the upper and lower layers. The sealed space can be surrounded by a supporting structure **346** (e.g., a toroidal gasket) that supports the sealed space at its upper and lower peripheral surfaces.

Referring to FIG. **11B**, piercing member **342** is shown as being depressed until the piercing member **342** has pierced both the upper and lower layers bringing the liquid into communication with the microfluidic network. A vent **346** adjacent the plunger allows gas trapped between the piercing member and the upper layer of the sealed space to escape without being forced into the microfluidic network.

Referring to FIG. **11C**, piercing member **342** is shown as fully actuated. A portion of the piercing member has displaced a corresponding volume of liquid from the sealed space and introduced the predetermined volume of liquid into the microfluidic device.

While the reservoirs have been described as having a sealed space that may be stationary with respect to a piercing member, other configurations are possible. For example, FIG. **12A** illustrates a reservoir having a sealed space **347**

that is secured with (e.g., integral with) respect to an actuation mechanism having a movable member **348** (e.g., a plunger) and a piercing member **349** supported by a piercing member support **350** that are stationary with respect to the sealed space. Typically, the sealed space is defined by a cavity within the movable member and a lower wall **351** that seals liquid within the sealed space. Piercing member is configured to rupture the lower wall when the movable member is depressed. Piercing member support has a shape generally complementary to the cavity of the movable member. Piercing member support includes a channel **352** connected to a microfluidic network to allow fluid released from the enclosed space to enter the microfluidic network.

Referring to FIG. **12B**, the movable member has been depressed so that the piercing member has just ruptured the lower layer of the sealed space. Referring to FIG. **12C**, the reservoir has been fully depressed onto the piercing member and piercing member support. The volume of fluid displaced from the reservoir generally corresponds to the volume of the piercing member support that enters the enclosed space. A channel **353** allows air displaced by the moveable member to exit.

While reservoirs have been described as having a piercing member that is secured with respect to some portion of the reservoir, other configurations are possible. For example, referring to FIG. **13**, a reservoir includes an actuation mechanism **354** (e.g., a piercing member such as a needle-like piercing member) that is unsecured with respect to the reservoir. A sealed space **355** of the reservoir is defined by an upper wall **356** and includes a channel **357** extending through a portion of a substrate **361** in which a microfluidic network is defined. A lower wall **358** of the sealed space separates the sealed space from a channel **359** of the microfluidic network. The piercing member occupies the channel **357** of the sealed space so that the piercing tip **360** of the piercing member rests against the lower wall **358**. Depressing the upper wall **356** of the reservoir drives the piercing member **354** through the lower wall and forces liquid within the sealed space into the microfluidic network.

As another example, FIGS. **14A** and **14B** illustrate a reservoir including an actuation mechanism (e.g., a piercing member) that is initially secured to an interior of an upper wall of the reservoir but separates at least partially from the upper wall upon actuation of the reservoir.

As yet another example, FIGS. **15A** and **15B** illustrate a reservoir including a piercing member **364** that is initially secured to an interior **365** of an upper wall **366** of the reservoir but substantially separates (e.g., completely separates) from the upper wall upon actuation of the reservoir.

While reservoirs have been described as having an enclosed space that is fixed or otherwise integral with a portion of the reservoir, other configurations are possible. For example, referring to FIG. **16**, a reservoir includes a capsule-like enclosed space **367** defined by an outer wall **368**. The outer wall is generally formed of a material having a low vapor transmission rate. Reservoir also includes an actuation mechanism having a moveable member **369** with a piercing member **370** that pierces the enclosed space to release liquid therein. The liquid passes along a channel **372** leading to a microfluidic network. A channel **371** allows gas (e.g., air) otherwise trapped by the movable member to exit.

While reservoirs have been described as generally overlying an inlet to a microfluidic network, other configurations are possible. For example, referring to FIG. **17**, a reservoir includes an enclosed space **373** in which liquid is stored and a connecting portion **374** connected to an inlet **376** of a microfluidic network. The enclosed space **373** and connect-

US 10,364,456 B2

27

ing portion **374** are separated by a rupturable seal **375** (e.g., a weak seal). In general, the rupturable seal **375** prevents liquid or vapor from exiting the enclosed space. However, upon the application of pressure to the liquid (e.g., by depressing a wall **377** of the enclosed space), the rupturable seal **375** ruptures allowing the liquid to pass through the weak seal to the connecting portion and into the microfluidic network **378**.

A still further embodiment of a reservoir with a piercing member is shown in FIG. **27A**, which shows a reservoir **2701** having an outer shell **2703** and a piercing element **2704** that are both made of the same piece of material. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2704** is generally conical in shape, with the apex adjacent to a membrane **2702**; its apex preferably does not exceed 0.040". The piercing element will puncture membrane **2702** and release liquid from reservoir **2701** when the outer shell is depressed. Representative dimensions are shown on FIG. **27A**. The reservoir may be constructed so that the upper surface is level, with a flat protective piece **2705** covering the base of the conical shape of piercing element **2704**.

Yet another embodiment of a reservoir with a piercing member is shown in FIG. **27B**, showing a reservoir **2711** having a single-piece outer shell **2712** and piercing element **2714**. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2714** can be frustoconical in shape, with its narrower side adjacent to membrane **2713**. Alternatively, piercing element **2714** can comprise several separate piercing elements, arranged within a conical space. Preferably there are four such piercing elements where multiple elements are present.

It is to be understood that the dimensions of the reservoir, piercing element, shell and moulding shown in FIGS. **27A** and **27B** as decimal quantities in inches are exemplary. In particular, the dimensions are such that the shell does not collapse under its own weight and is not so as strong to prohibit depression of the piercing member when required during operation of the device.

Furthermore, the materials of the various embodiments are also chosen so that the device has a shelf-life of about a year. By this it is meant that the thickness of the various materials are such that they resist loss, through means such as diffusion, of 10% of the liquid volume contained therein over a desired shelf-life period.

Preferably the volume of the reservoir is around 150 μ l before a shell is depressed. Upon depression of a shell, the volume is preferably deformed to around half its original volume.

While devices for processing samples have been described as having a generally planar configuration, other configurations can be used. For example, referring to FIG. **18**, a device **700** configured to process a polynucleotide-containing sample, such as to prepare the sample for amplification of the polynucleotides, has a generally tube-like or vial-like configuration. Device **700** includes a sample reservoir **704**, a reagent reservoir **706**, a gas pressure generator **708**, a closure (e.g., a cap **710**), and a processing region **702** including a retention member **704** having a plurality of particles (e.g. carboxylate beads **705** surface-modified with a ligand, e.g., poly-L-lysine and/or poly-D-lysine, or poly-ethyleneimine). Retention member **705** and beads **705** may share any or all properties of retention member **216** and

28

surface-modified particles **218**. Device **700** also includes an opening **716** and a valve, e.g., a thermally actuated valve **714** for opening and closing opening **716**.

In use, a polynucleotide-containing sample is added to sample reservoir **704**. Typical sample amounts range from about 100 μ L to about 2 mL, although greater or smaller amounts may be used.

Reagent reservoir **706** may be provided to users of device **700** with pre-loaded reagent. Alternatively, device **700** may be configured so that users add reagent to device **700**. In any event, the reagents may include, e.g., NaOH solutions and/or buffer solutions such as any of such solutions discussed herein.

Once sample and, if necessary, reagent have been added to device **700**, cap **710** is closed to prevent evaporation of sample and reagent materials.

Referring also to FIG. **19**, an operator **718** is configured to operate device **700**. Operator **718** includes a first heat source **720** and a second heat source **722**. First heat source **720** heats sample present within sample reservoir **704**, such as to lyse cells of the polynucleotide-containing sample to prepare free polynucleotides.

Device **700** may also include an enzyme reservoir **712** comprising an enzyme, e.g., a protease such as pronase, configured to cleave peptide bonds of polypeptides present in the polynucleotide-containing sample. Enzyme reservoir **712** may be provided to users of device **700** with pre-loaded enzyme. Alternatively, device **700** may be configured so that users add enzyme to device **700**.

Device **700** may be used to reduce the amount of inhibitors present relative to the amount of polynucleotides to be determined. Thus, the sample is eluted through processing region **702** to contact constituents of the sample with beads **705**. Beads **705** retain polynucleotides of the sample as compared to inhibitors as described elsewhere herein. With valve **714** in the open state, sample constituents not retained in processing region **702** exit device **700** via the opening.

Once the polynucleotide-containing sample has eluted through processing region **702**, an amount of reagent, e.g., a wash solution, e.g., a buffer such as Tris-EDTA pH 8.0 with 1% Triton X 100 is eluted through processing region **702**. The wash solution is generally stored in reagent reservoir **706**, which may include a valve configured to release an amount of wash solution. The wash solution elutes remaining polynucleotide-containing sample and inhibitors without eluting retained polynucleotides.

Once inhibitors have been separated from retained polynucleotides, the polynucleotides are released from beads **705**. In some embodiments, polynucleotides are released by contacting the beads **705** with a release solution, e.g., a NaOH solution or buffer solution having a pH different from that of the wash solution. Alternatively, or in combination, beads **705** with retained polynucleotides are heated, such as by using second heat source **722** of operator **718**. When heat is used to release the polynucleotides, the release solution may be identical with the wash solution.

Gas pressure generator **708** may be used to expel an amount of release solution with released polynucleotides from device **700**. Gas pressure generator and/or operator **718** may include a heat source to heat gas present within generator **708**. The heated gas expands and provides the gas pressure to expel sample. In some embodiments, and whether or not thermally generated gas pressure is used, gas pressure generator **708** is configured to expel a predetermined volume of material. Typically, the amount of expelled

US 10,364,456 B2

29

solution is less than about 500 μL , less than about 250 μL , less than about 100 μL , less than about 50 μL , e.g., less than about 25 μL .

EXAMPLES

The following Examples are illustrative and are not intended to be limiting.

Example 1 Preparing Retention Member

Carboxylate surface magnetic beads (Sera-Mag Magnetic Carboxylate modified, Part #3008050250, Seradyn) at a concentration of about 1011 mL⁻¹ were activated for 30 minutes using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in a pH 6.1 500 mM 2-(N-Morpholinio)-ethanesulfonic acid (MES) buffer solution. Activated beads were incubated with 3000 Da or 300,000 Da average molecular weight poly-L-lysine (PLL). After 2 washes to remove unbound PLL, beads were ready for use.

Example 2 Microfluidic Device

Referring to FIGS. 20 and 21, a microfluidic device 300 was fabricated to demonstrate separation of polynucleotides from inhibitors. Device 300 comprises first and second substrate portions 302', 304', which respectively comprise first and second layers 302 a', 302 b' and 304 a', 304 b'. First and second layers 302 a', 302 b' define a channel 306' comprising an inlet 310' and an outlet 312'. First and second layers 304 a', 304 b' define a channel 308' comprising an inlet 314' and an outlet 316'. First and second substrate portions 302', 304' were mated using adhesive 324' so that outlet 312' communicated with inlet 314' with a filter 318' positioned therebetween. A portion of outlet 312' was filed with the activated beads prepared above to provide a processing region 320' comprising a retention member (the beads). A pipette 322' (FIG. 22) secured by adhesive 326' facilitated sample introduction.

In use, sample introduced via inlet 310' passed along channel and through processing region 320'. Excess sample material passed along channel 308' and exited device 300' via outlet 316'. Polynucleotides were preferentially retained by the beads as compared to inhibitors. Once sample had been introduced, additional liquids, e.g., a wash liquid and/or a liquid for use in releasing the retained polynucleotides were introduced via inlet 326'.

Example 3 Retention of DNA

Retention of polynucleotides by the poly-L-lysine modified beads of device 300' was demonstrated by preparing respective devices comprising processing regions having a volume of about 1 μL including about 1000 beads. The beads were modified with poly-L-lysine of between about 15,000 and 30,000 Da. Each processing region was filled with a liquid comprising herring sperm DNA (about 20 μL of sample with a concentration of about 20 mg/mL) thereby placing the beads and liquid in contact. After the liquid and beads had been in contact for 10 minutes, the liquid was removed from each processing region and subjected to quantitative real-time PCR to determine the amount of herring sperm DNA present in the liquid.

Two controls were performed. First, an otherwise identical processing region was packed with unmodified beads, i.e., beads that were identical with the poly-L-lysine beads

30

except for the activation and poly-L-lysine incubation steps. The liquid comprising herring sperm DNA was contacted with these beads, allowed to stand for 10 minutes, removed, and subjected to quantitative real-time PCR. Second, the liquid comprising the herring sperm DNA ("the unprocessed liquid") was subjected to quantitative real-time PCR.

Referring to FIG. 22, the first and second controls exhibited essentially identical responses indicating the presence of herring sperm DNA in the liquid contacted with the unmodified beads and in the unprocessed liquid. The liquid that had contacted the 3,000 poly-L-lysine beads exhibited a lower response indicating that the modified beads had retained substantially all of the herring sperm DNA. The PCR response of the liquid that had contacted the 300,000 Da poly-L-lysine beads exhibited an amplification response that was at least about 50% greater than for the 3,000 Da beads indicating that the lower molecular weight surface modification was more efficient at retaining the herring sperm DNA.

Example 4 Releasing DNA From Poly-L-Lysine Modified Beads

Devices having processing regions were packed with 3,000 Da poly-L-lysine modified beads. Liquid comprising polynucleotides obtained from group B streptococci (GBS) was contacted with the beads and incubated for 10 minutes as above for the herring sperm DNA. This liquid had been obtained by subjecting about 10,000 GBS bacteria in 10 μL of 20 mM Tris pH 8, 1 mM EDTA, 1% Triton X-100 buffer to thermal lysing at 97° C. for 3 min.

After 10 minutes, the liquid in contact with the beads was removed by flowing about 10 μL of wash solution (Tris-EDTA pH 8.0 with 1% Triton X 100) through the processing region. Subsequently, about 1 μL of 5 mM NaOH solution was added to the processing region. This process left the packed processing region filled with the NaOH solution in contact with the beads. The solution in contact with the beads was heated to 95° C. After 5 minutes of heating at 95° C., the solution in contact with the beads was removed by eluting the processing region with a volume of solution equal to three times the void volume of the processing region.

Referring to FIG. 23, five aliquots of solution were subjected to quantitative real-time PCR amplification. Aliquots E1, E2, and E3 each contained about 1 μL of liquid. Aliquot L corresponds to liquid of the original sample that had passed through the processing region. Aliquot W was liquid obtained from wash solution without heating. Aliquot E1 corresponds to the dead volume of device 300, about equal to the volume of channel 308. Thus, liquid of aliquot E1 was present in channel 308 and not in contact with the beads during heating. This liquid had passed through the processing region prior to heating. Aliquot E2 comprises liquid that was present within the processing region and in contact with the beads during heating. Aliquot E3 comprises liquid used to remove aliquot E2 from the processing region.

As seen in FIG. 23, more than 65% of the GBS DNA present in the initial sample was retained by and released from the beads (Aliquot E2). Aliquot E2 also demonstrates the release of more than 80% of the DNA that had been retained by the beads. Less than about 18% of the GBS DNA passed through the processing region without being cap-

tured. The wash solution without heating comprised less than 5% of the GBS DNA (Aliquot W).

Example 5 Separation of Polynucleotides and Inhibitors

Buccal cells from the lining of the cheeks provide a source of human genetic material (DNA) that may be used for single nucleotide polymorphism (SNP) detection. A sample comprising buccal cells was subjected to thermal lysing to release DNA from within the cells. Device 300 was used to separate the DNA from concomitant inhibitors as described above. A cleaned-up sample corresponding to aliquot E2 of FIG. 23 was subjected to polymerase chain reaction. A control or crude sample as obtained from the thermal lysing was also amplified.

Referring to FIG. 24, the cleaned-up sample exhibited substantially higher PCR response in fewer cycles than did the control sample. For example, the clean-up sample exceeded a response of 20 within 32 cycles whereas the control sample required about 45 cycles to achieve the sample response.

Blood acts as a sample matrix in variety of diagnostic tests including detection of infectious disease agents, cancer markers and other genetic markers. Hemoglobin present in blood samples is a documented potent inhibitor of PCR. Two 5 ml blood samples were lysed in 20 mM Tris pH 8, 1 mM EDTA, 1% SDS buffer and introduced to respective devices 300, which were operated as described above to prepare two clean-up samples. A third 5 ml blood sample was lysed and prepared using a commercial DNA extraction method Puregene, Gentra Systems, MN. The respective cleaned-up samples and sample subjected to the commercial extraction method were used for a Allelic discrimination analysis (CYP2D6*4 reagents, Applied Biosystems, CA). Each sample contained an amount of DNA corresponding to about 1 ml of blood.

Referring to FIG. 25, the cleaned-up and commercially extracted samples exhibited similar PCR response demonstrating that the processing region of device 300' efficiently removed inhibitors from the blood samples.

Example 6 Protease Resistant Retention Member

The preparation of polynucleotide samples for further processing often includes subjecting the samples to protease treatment in which a protease cleaves peptide bonds of proteins in the sample. An exemplary protease is pronase, a mixture of endo- and exo-proteases. Pronase cleaves most peptide bonds. Certain ligands, such as poly-L-lysine are susceptible to rupture by pronase and other proteases. Thus, if samples are generally not subjected to protease treatment in the presence of the retention member if the ligands bound thereto are susceptible to the proteases.

Poly-D-lysine, the dextro enantiomer of poly-lysine resists cleavage by pronase and other proteases. The ability of a retention member comprising bound poly-D-lysine to retain DNA even when subjected to a protease treatment was studied.

Eight (8) samples were prepared. A first group of 4 samples contained 1000 GBS cells in 10 μ l buffer. A second group of 4 samples contained 100 GBS cells in 10 μ l buffer. Each of the 8 samples was heated to 97° C. for 3 min to lyse the GBS cells. Four (4) sample sets were created from the heated samples. Each sample set contained 1 sample from each of the first and second groups. The samples of each sample sets were treated as follows.

Referring to FIG. 26A, the samples of sample set 1 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 2 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 3 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 4 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

As seen in FIG. 26B, an average of more than 80% of DNA from the GBS cells was recovered using sample set 4 in which the samples were contacted with poly-D-lysine modified beads and subjected to pronase incubation in the presence of the beads without protease inactivation. The

US 10,364,456 B2

33

recovery efficiency for sample set 4 is more than twice as high as for any of the other samples. Specifically, the recovery efficiencies for sample sets 1, 2, 3, and 4, were 29%, 32%, 14%, and 81.5%, respectively. The efficiencies demonstrate that high recovery efficiencies can be obtained for samples subjected to protease incubation in the presence of a retention member that retains DNA.

Other embodiments are within the claims.

What is claimed is:

1. A method for processing polynucleotide-containing sample, the method comprising:

retaining polynucleotides from a sample on a plurality of binding particles in a process chamber under a first set of conditions, wherein the retaining step comprises binding the polynucleotides to the surfaces of the plurality of binding particles comprising a poly-cationic substance, wherein the sample has a volume from 0.5 microliters to 3 milliliters;

wherein the first set of conditions includes a first pH of about 8.5 or less and a first temperature, wherein the first temperature is about 50° C.;

releasing the polynucleotide from the plurality of binding particles under a second set of conditions; and

wherein the second set of conditions includes increasing the pH to a second pH by addition of a hydroxide solution and increasing the temperature to a second temperature, wherein the second temperature is between about 80° C. and about 100° C.

2. The method of claim 1, wherein the second pH is about 11.3 or greater.

3. The method of claim 1, wherein the poly-cationic substance is covalently bound to the surfaces of the binding particles.

4. The method of claim 1, wherein the binding particles comprise one or more carboxylic groups to provide an attachment point for the poly-cationic substance.

5. The method of claim 1, wherein the second temperature is between about 90° C. and about 100° C.

6. The method of claim 1, wherein the heating step comprises heating the plurality of binding particles in the presence of a liquid, wherein the second temperature is insufficient to boil the liquid.

7. The method of claim 1, further comprising maintaining the second temperature for between about 1 and 10 minutes.

8. The method of claim 1, wherein the second temperature is maintained for between about 1 and 7 minutes.

9. The method of claim 1, wherein the sample is heated for about 15 minutes or less while contacting the binding particles with the basic solution thereby releasing the polynucleotides.

10. The method of claim 1, wherein the poly-cationic substance includes polyethyleneimine (PEI).

11. The method of claim 1, wherein the poly-cationic substance has a molecular weight between 600-800 DA.

34

12. The method of claim 1, wherein the ratio of the volume of original sample moved through the processing region to the volume of liquid into which the polynucleotides are released is at least about 10.

13. The method of claim 1, wherein the particles occupy about 75 percent or less of the total volume of the process chamber.

14. A method for processing polynucleotide-containing samples, the method comprising:

retaining one or more polynucleotides from a sample on a plurality of binding particles under a first set of conditions, wherein a surface of one or more binding particles is modified with a poly-cationic material, wherein the sample has a volume from 0.5 microliters to 3 milliliters;

wherein the first set of conditions includes a first pH of 8.5 or less and a first temperature of about 50° C.;

releasing the polynucleotide from the plurality of binding particles under a second set of conditions; and

wherein the second set of conditions includes increasing the pH by at least three units by addition of a hydroxide solution and increasing the temperature by at least about 40° C. to a second temperature of at least about 90° C.

15. The method of claim 14, wherein the pH of the second set of conditions is about 11.3 or greater.

16. The method of claim 14, wherein the poly-cationic material includes polyethyleneimine (PEI).

17. The method of claim 14, wherein the ratio of the volume of original sample moved through the processing region to the volume of liquid into which the polynucleotides are released is at least about 10.

18. A method for processing a polynucleotide-containing sample, the method comprising:

contacting the sample with a plurality of binding particles, the binding particles retaining one or more polynucleotides thereon at a first pH and a first temperature, wherein the sample has a volume from 0.5 microliters to 3 milliliters, wherein the first temperature is about 50° C., wherein a surface of one or more binding particles is modified with a poly-cationic polyimide or polyethyleneimine (PEI); and

contacting the binding particles with a basic hydroxide solution at a second pH and a second temperature, the second temperature greater than the first temperature, thereby releasing the polynucleotides from the plurality of binding particle, wherein the second temperature is between about 80° C. and about 100° C.

19. The method of claim 18, wherein the first pH is about 8.5 or less.

20. The method of claim 18, wherein the second pH is about 11.3 or greater.

* * * * *

EXHIBIT 44



US010443088B1

(12) **United States Patent**
Wu et al.

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(45) **Date of Patent:** ***Oct. 15, 2019**

(54) **METHOD FOR PROCESSING
POLYNUCLEOTIDE-CONTAINING SAMPLES**

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See application file for complete search history.

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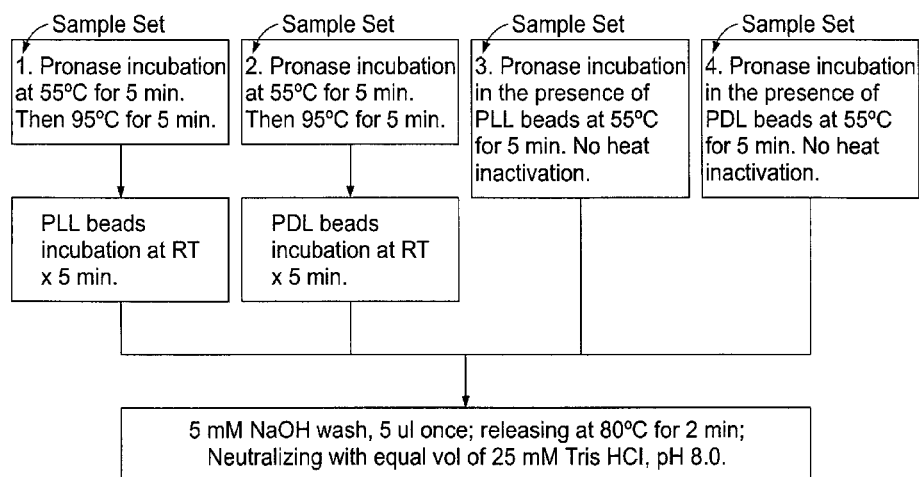
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(57) ABSTRACT

Methods and systems for processing polynucleotides (e.g., DNA) are disclosed. A processing region includes one or more surfaces (e.g., particle surfaces) modified with ligands that retain polynucleotides under a first set of conditions (e.g., temperature and pH) and release the polynucleotides under a second set of conditions (e.g., higher temperature and/or more basic pH). The processing region can be used to, for example, concentrate polynucleotides of a sample and/or separate inhibitors of amplification reactions from the polynucleotides. Microfluidic devices with a processing region are disclosed.

21 Claims, 25 Drawing Sheets



US 10,443,088 B1

Page 2

Related U.S. Application Data

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Page 11

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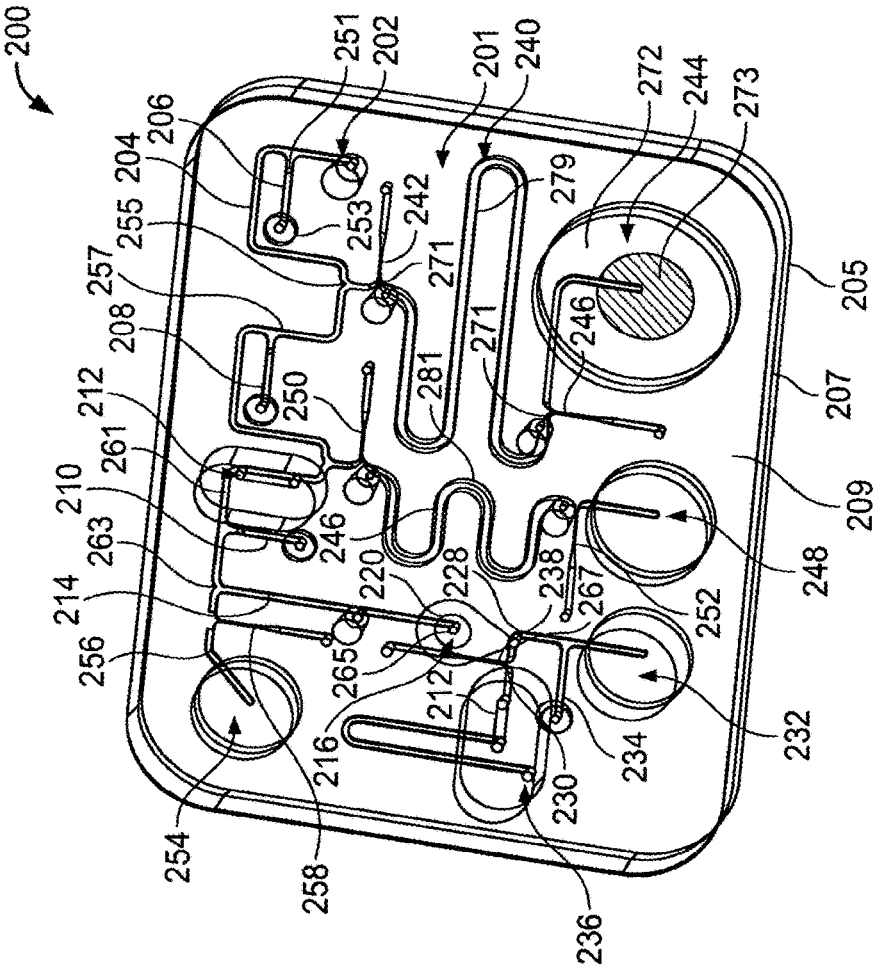


FIG. 1

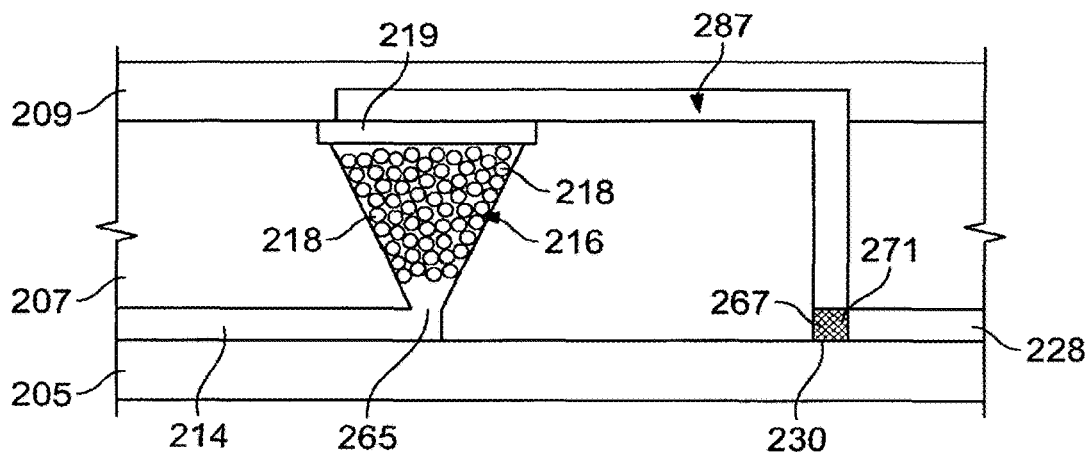


FIG. 2

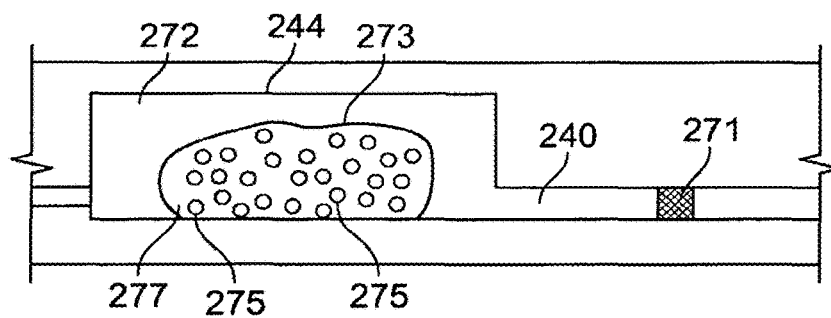


FIG. 3

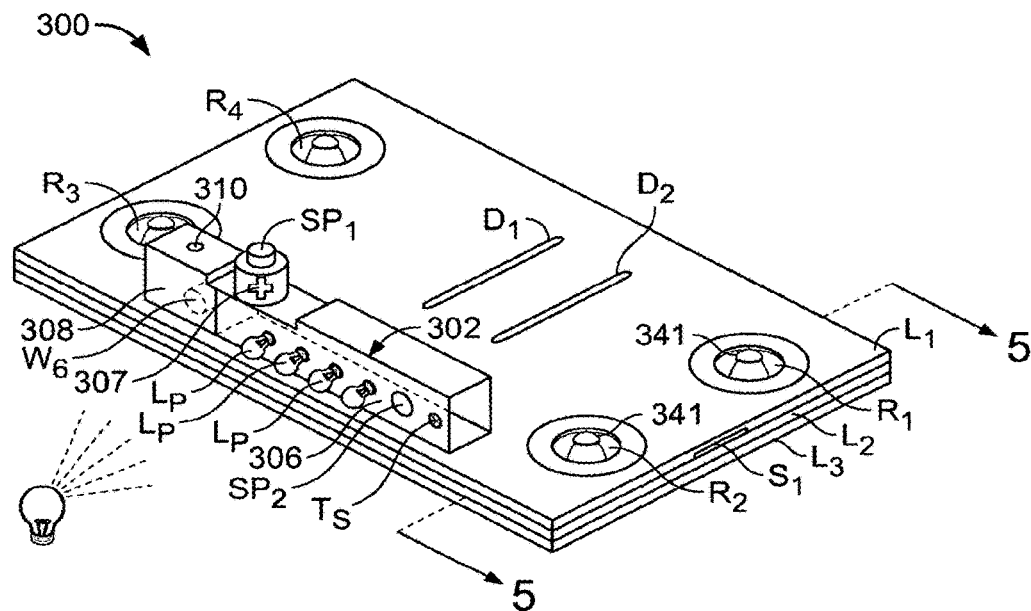


FIG. 4

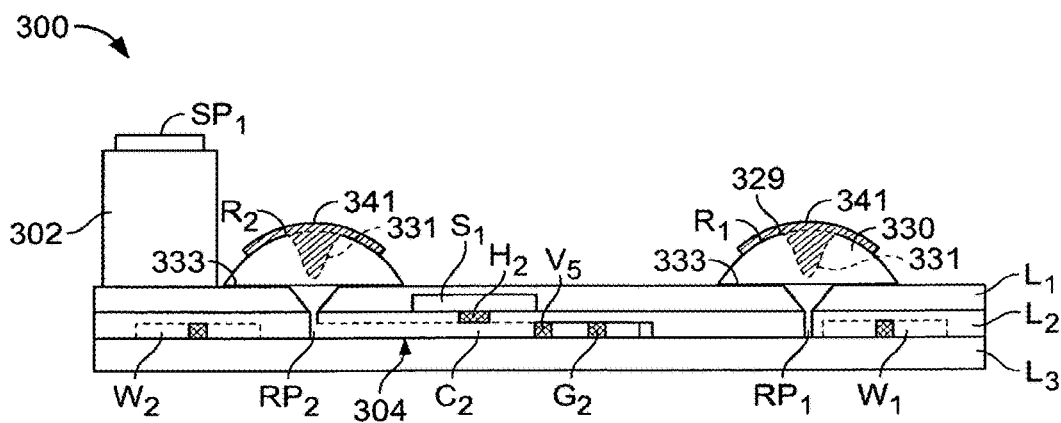
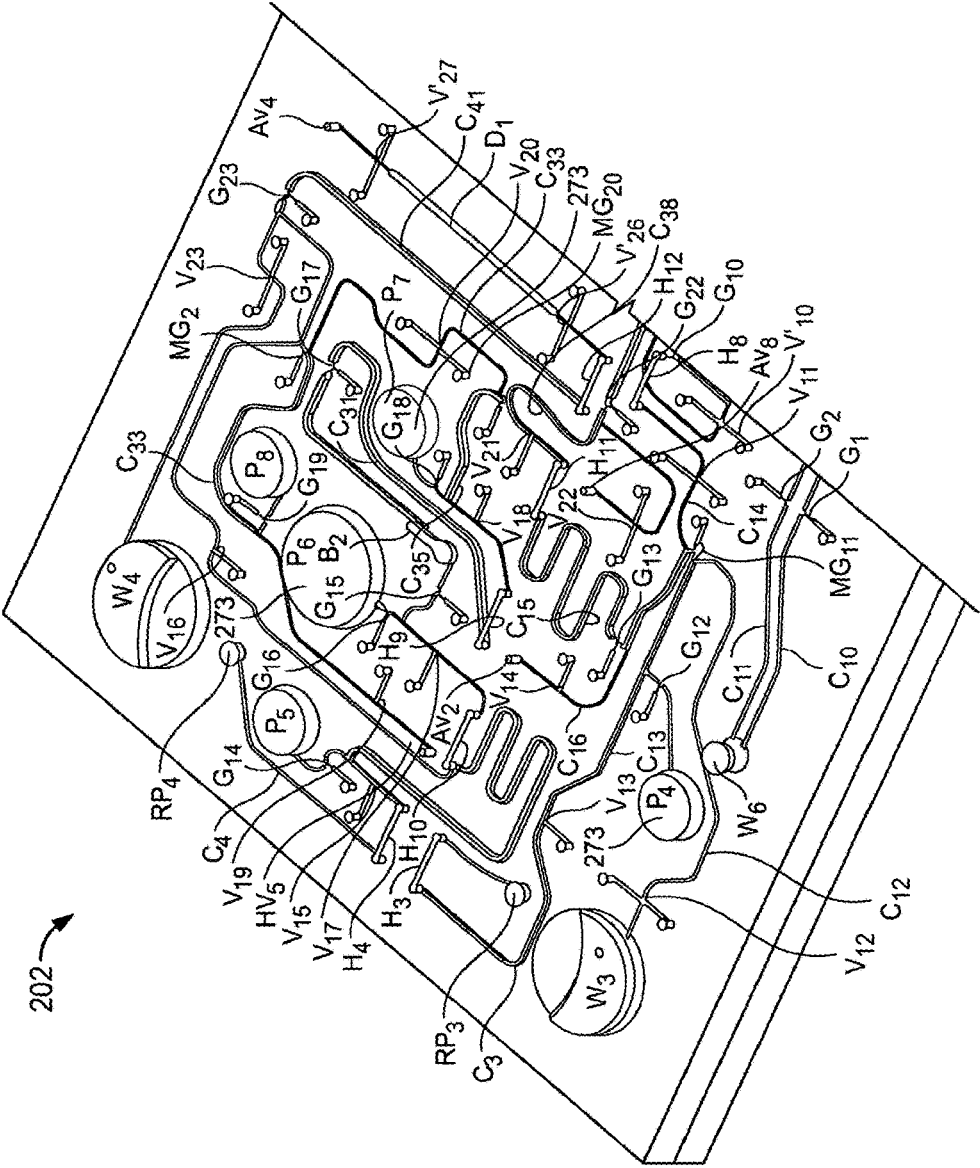


FIG. 5



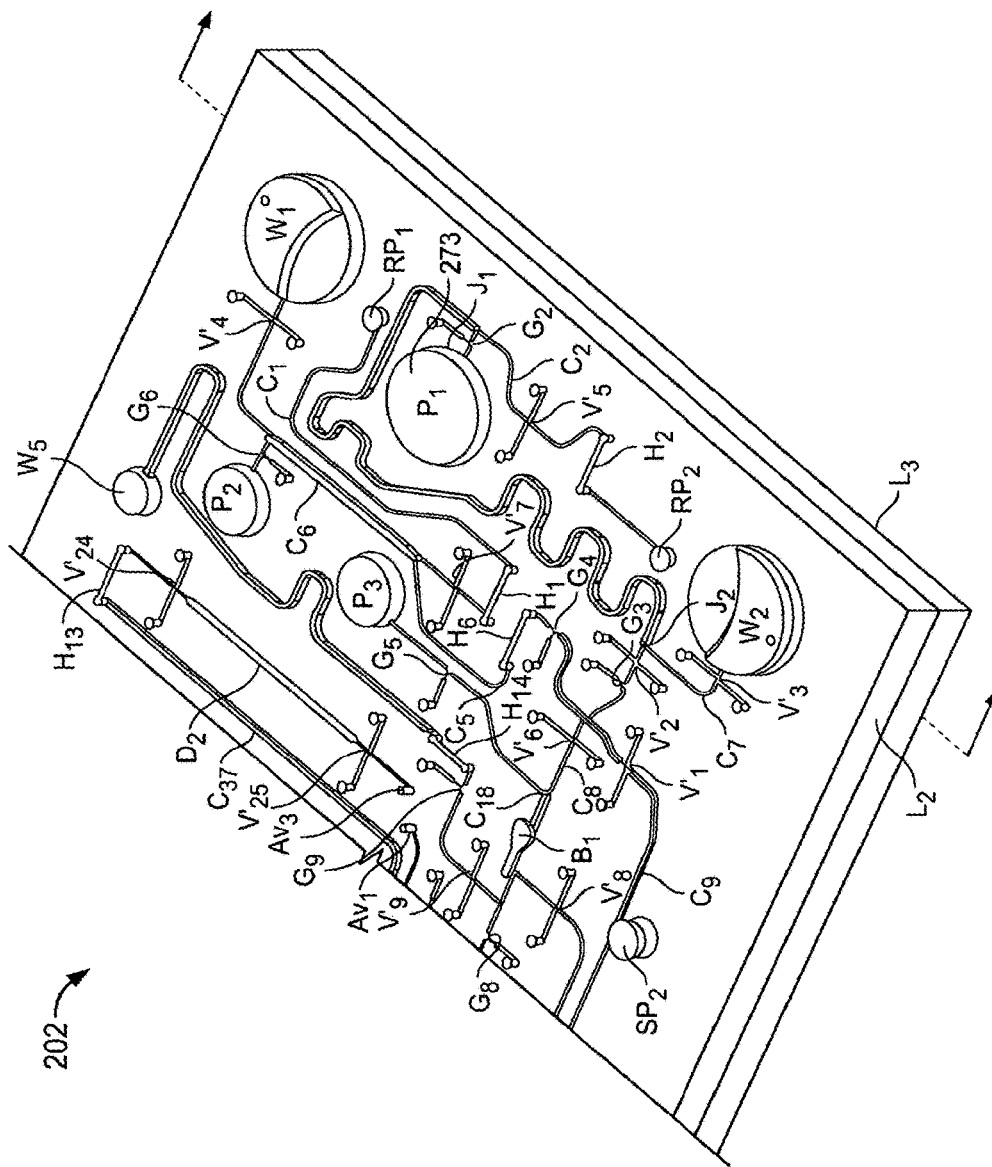


FIG. 6B

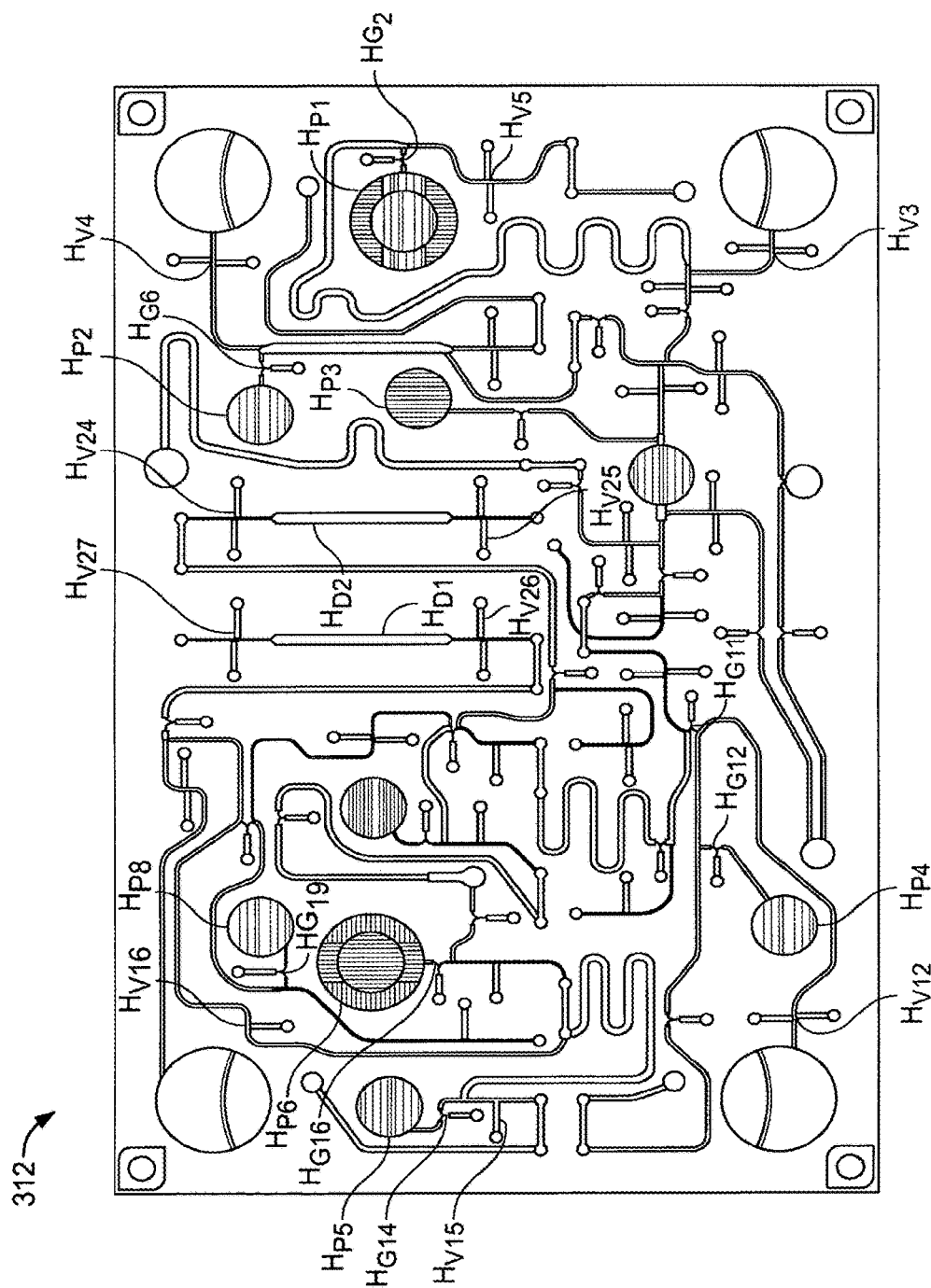


FIG. 7

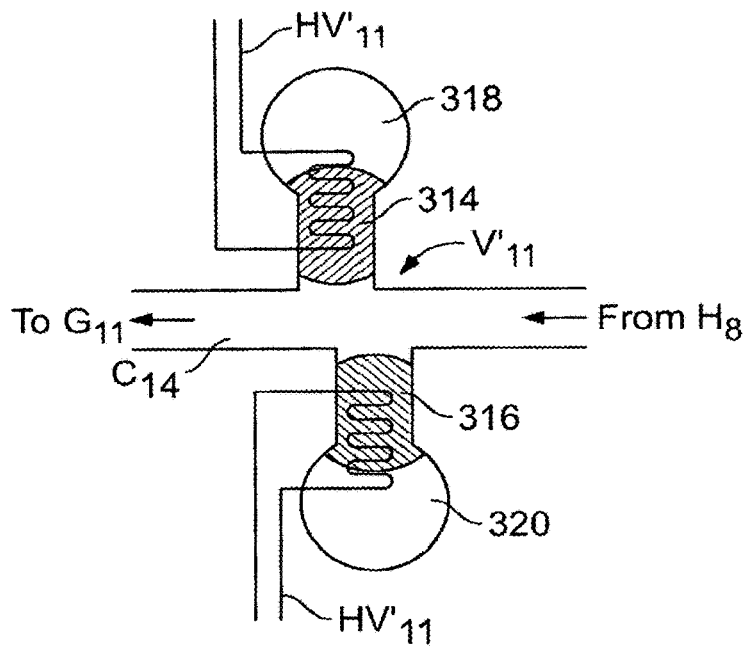


FIG. 8

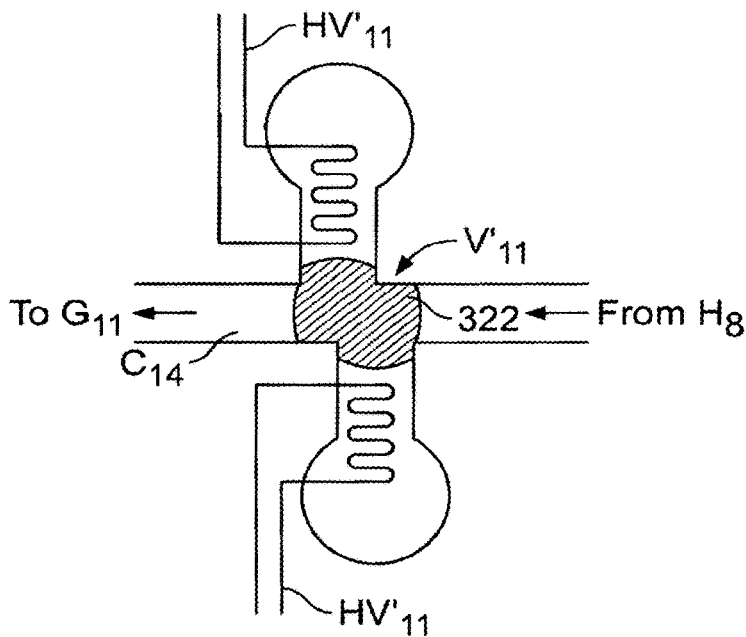


FIG. 9

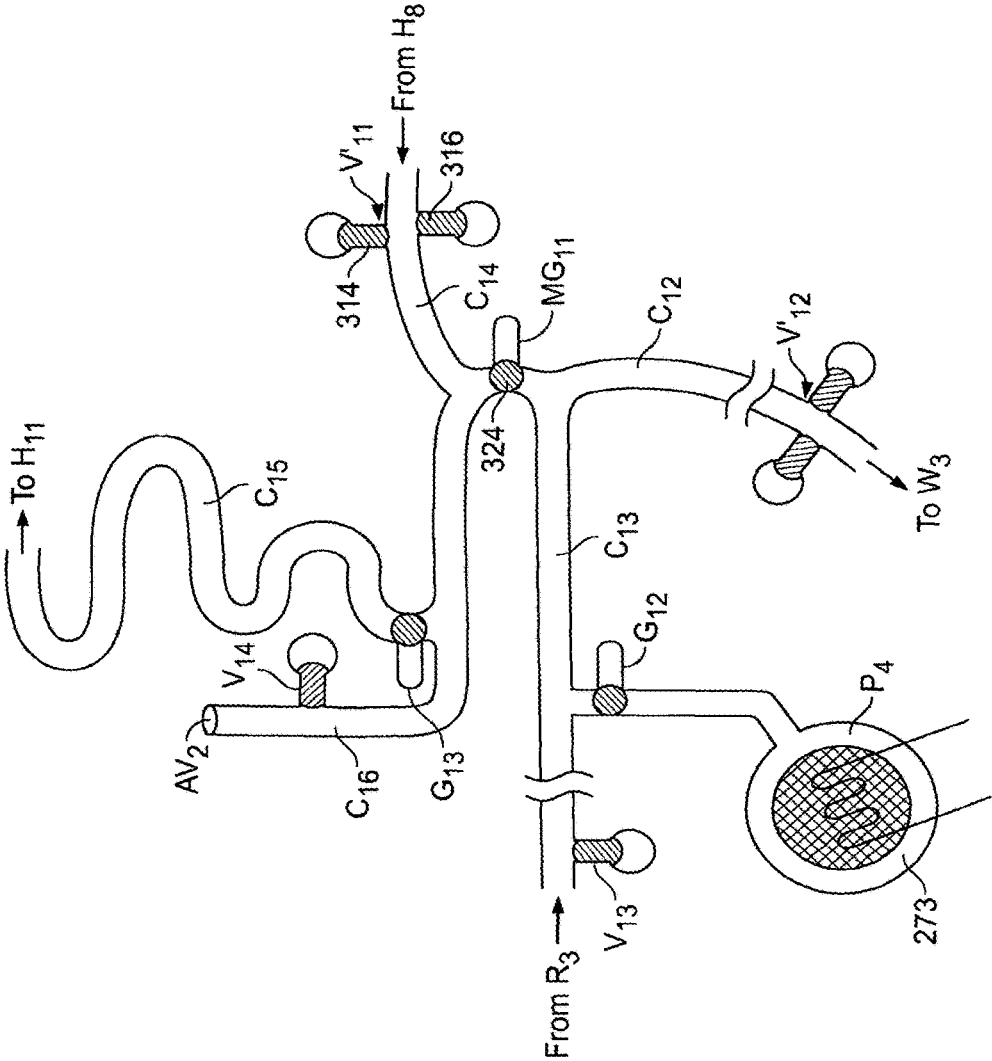


FIG. 10A

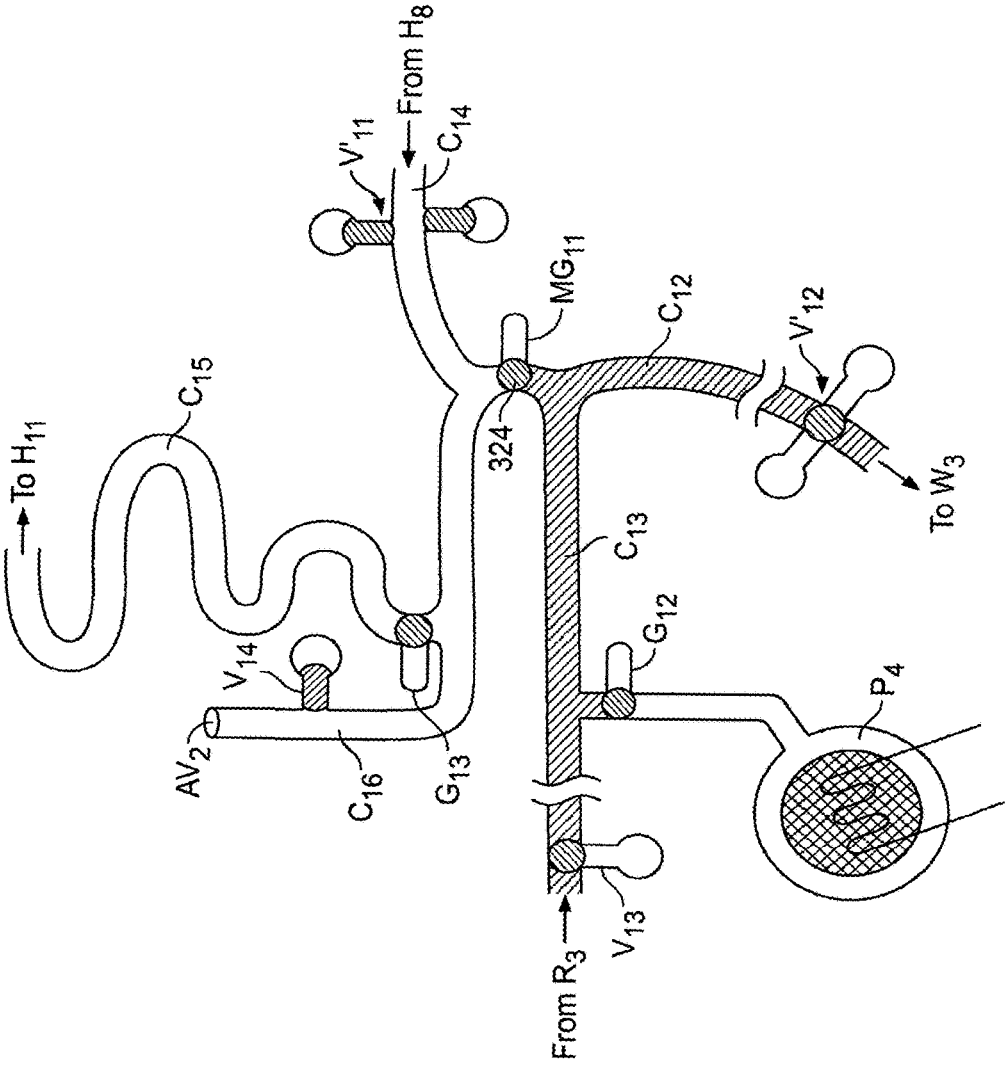


FIG. 10B

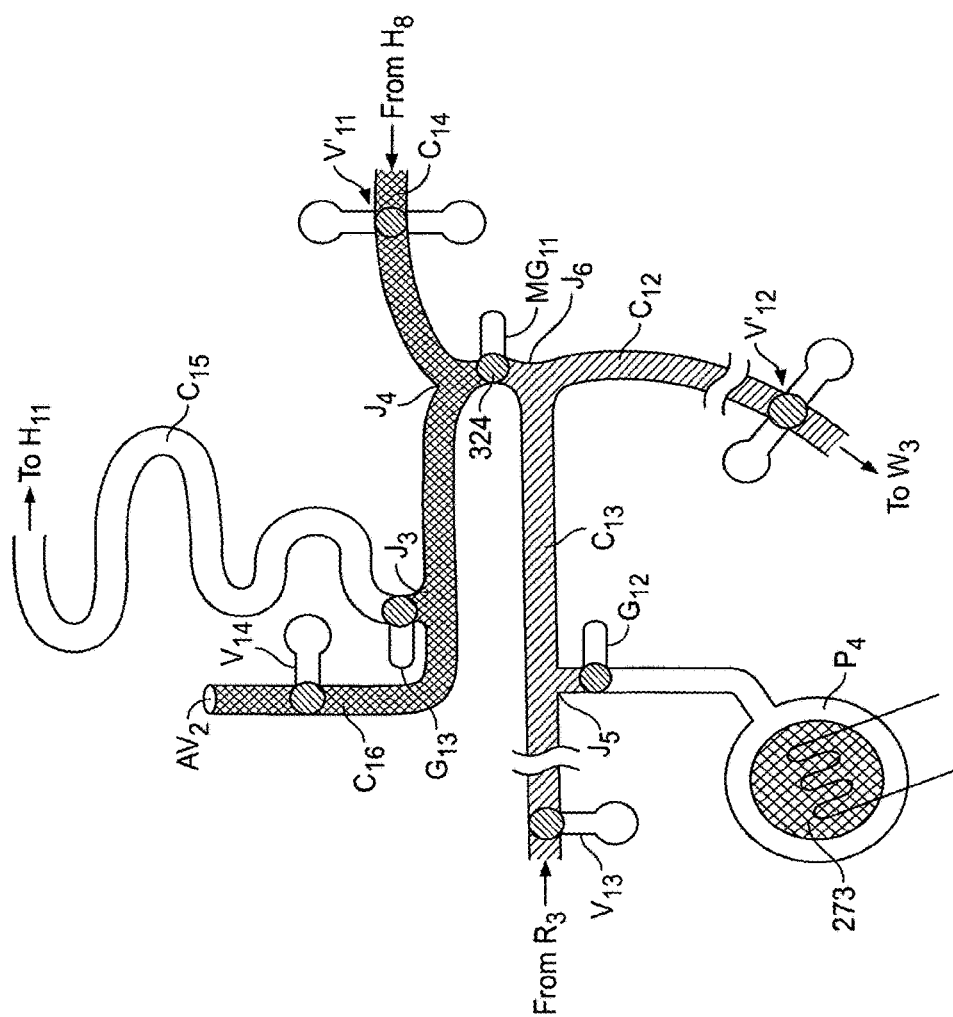


FIG. 10C

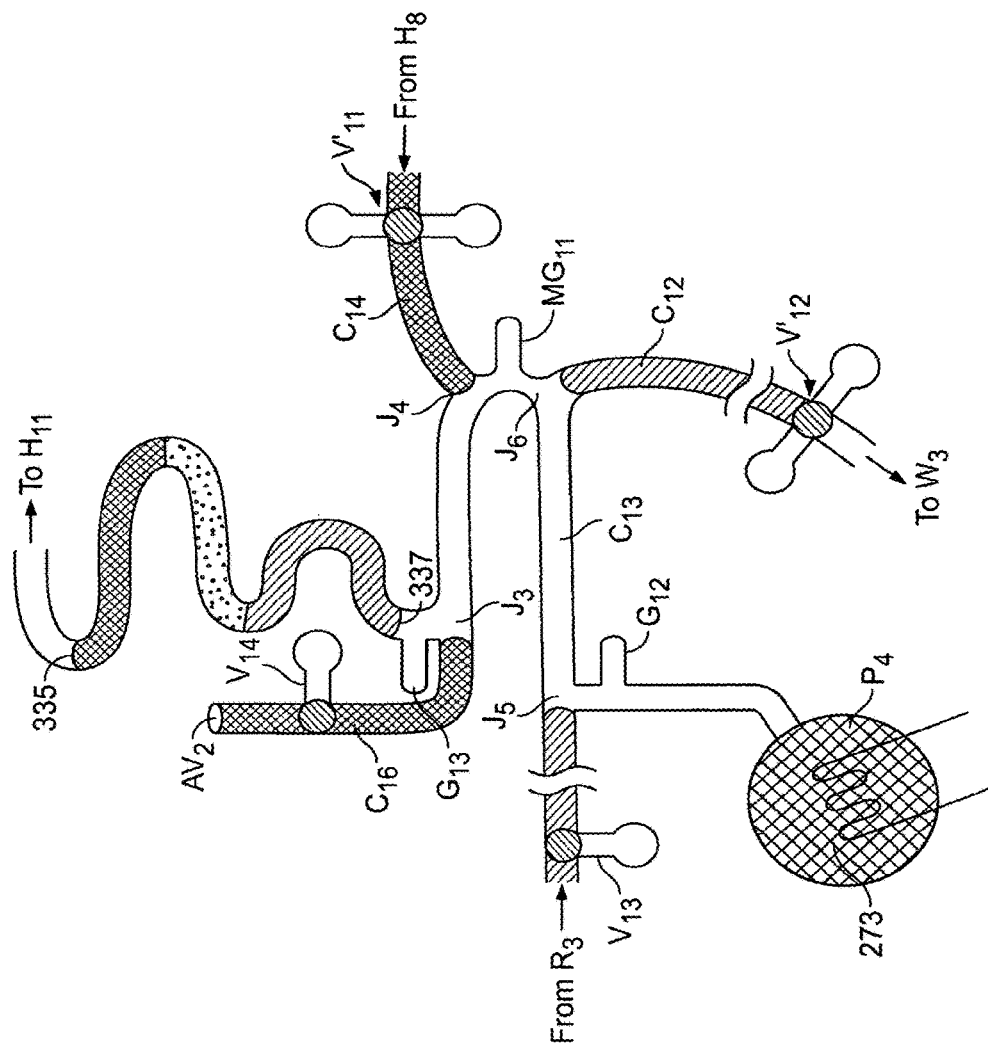


FIG. 10D

U.S. Patent

Oct. 15, 2019

Sheet 12 of 25

US 10,443,088 B1

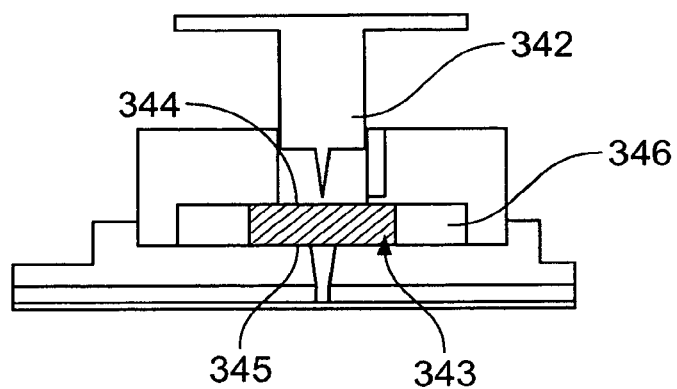


FIG. 11A

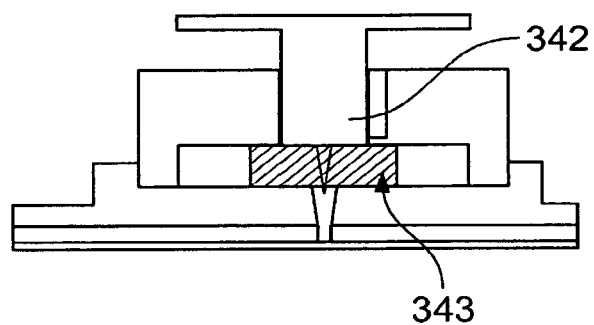


FIG. 11B

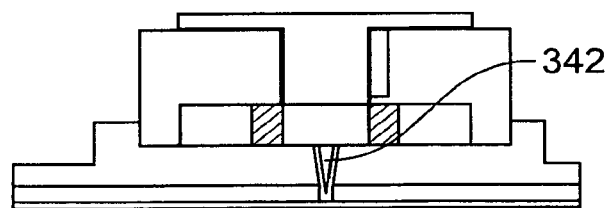


FIG. 11C

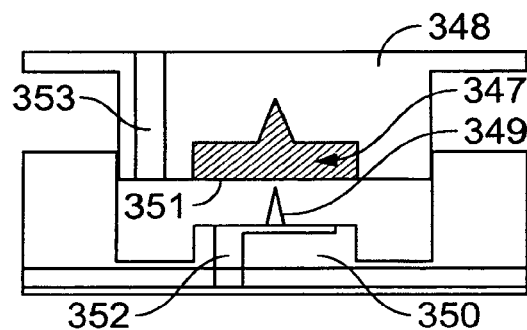


FIG. 12A

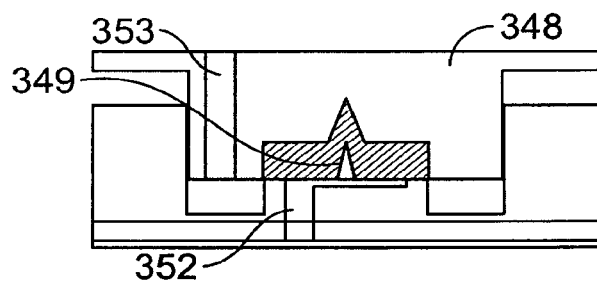


FIG. 12B

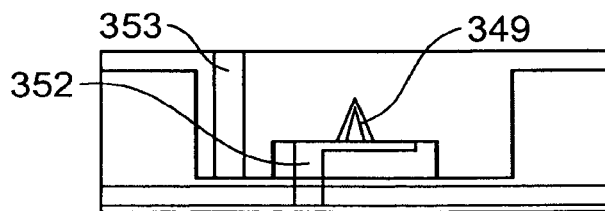


FIG. 12C

U.S. Patent

Oct. 15, 2019

Sheet 14 of 25

US 10,443,088 B1

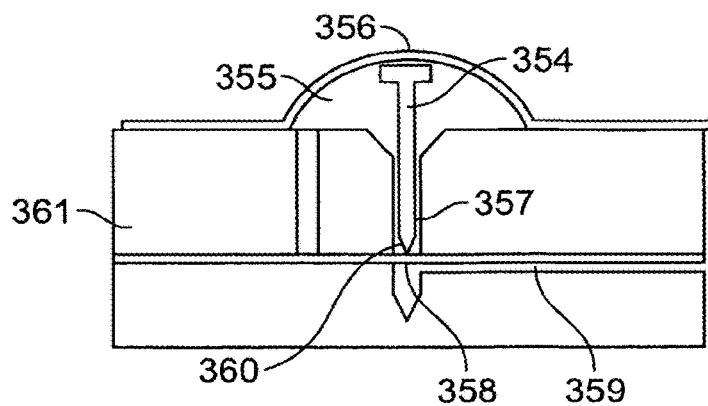


FIG. 13

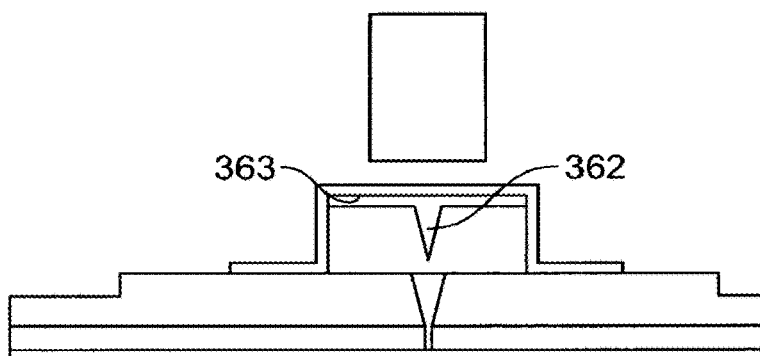


FIG. 14A

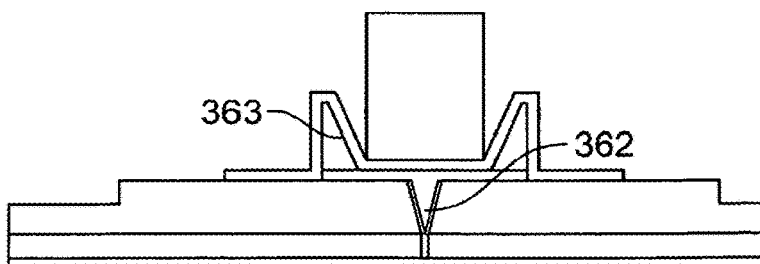


FIG. 14B

U.S. Patent

Oct. 15, 2019

Sheet 15 of 25

US 10,443,088 B1

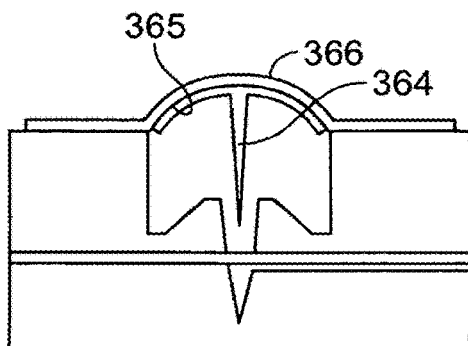


FIG. 15A

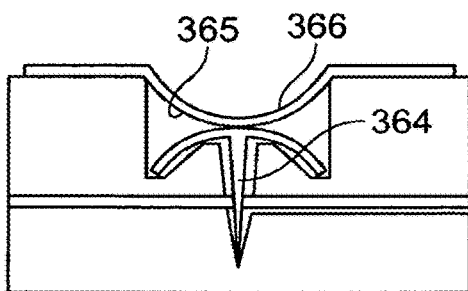


FIG. 15B

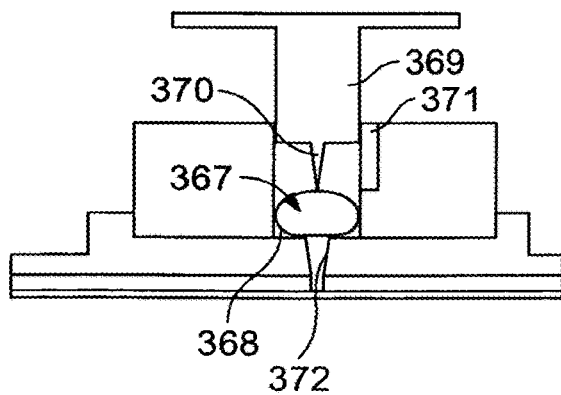


FIG. 16

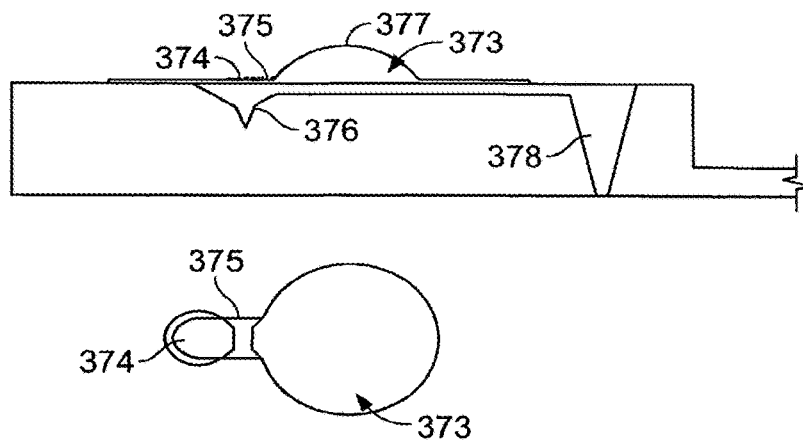


FIG. 17

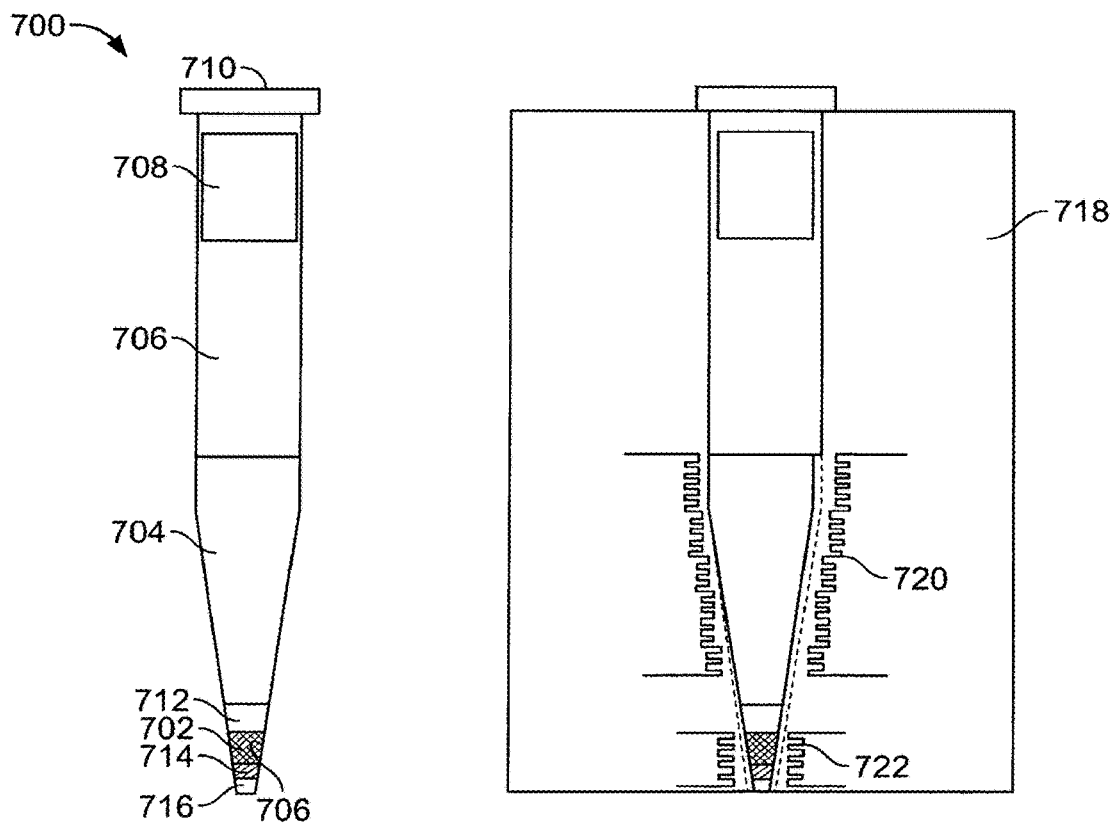


FIG. 18

FIG. 19

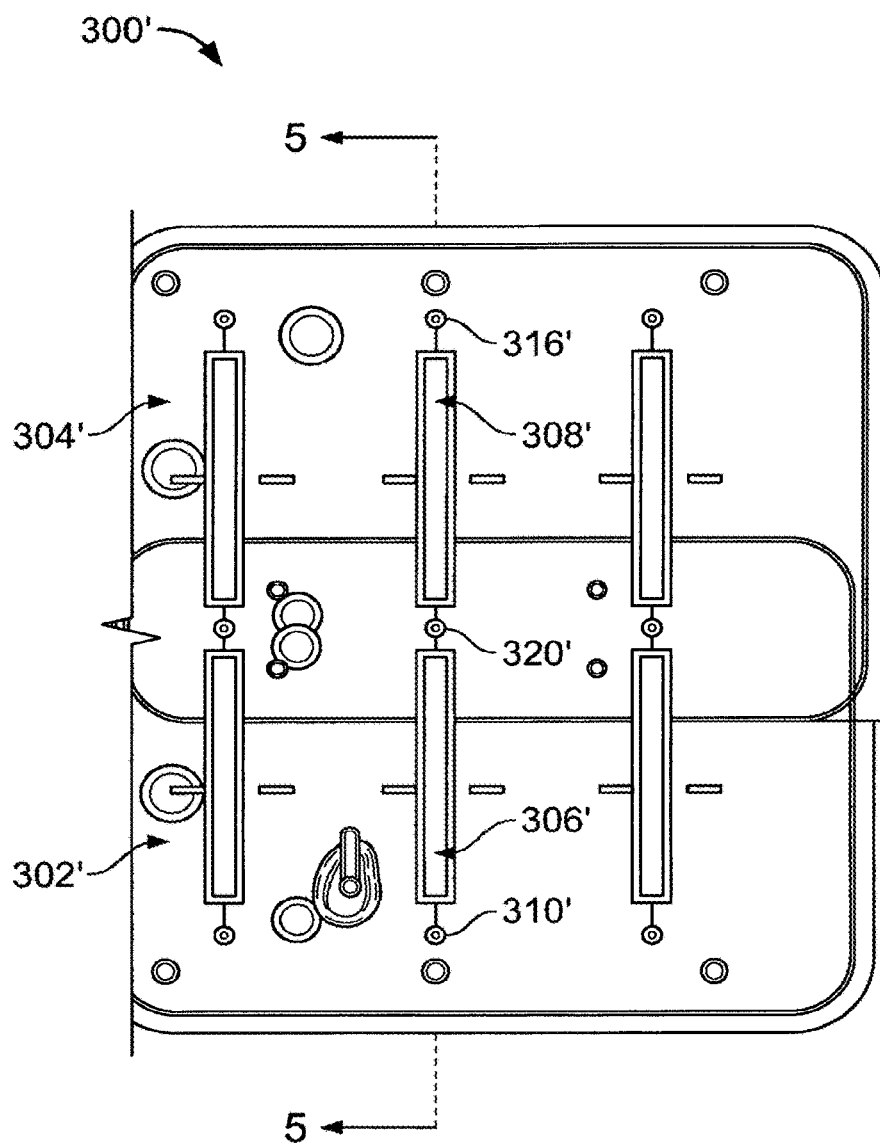


FIG. 20

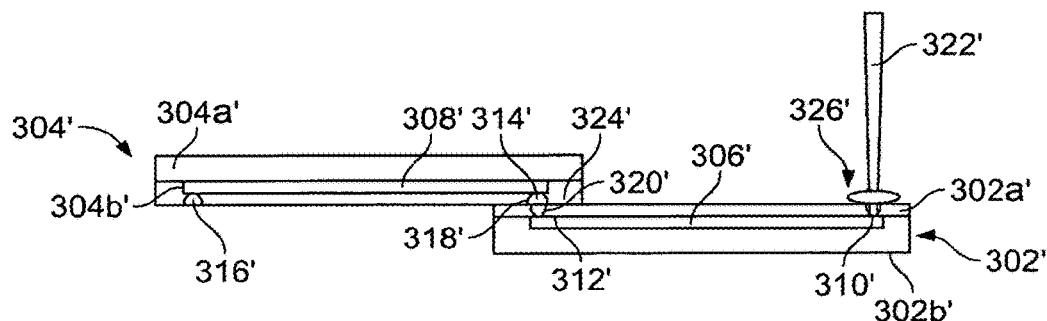


FIG. 21

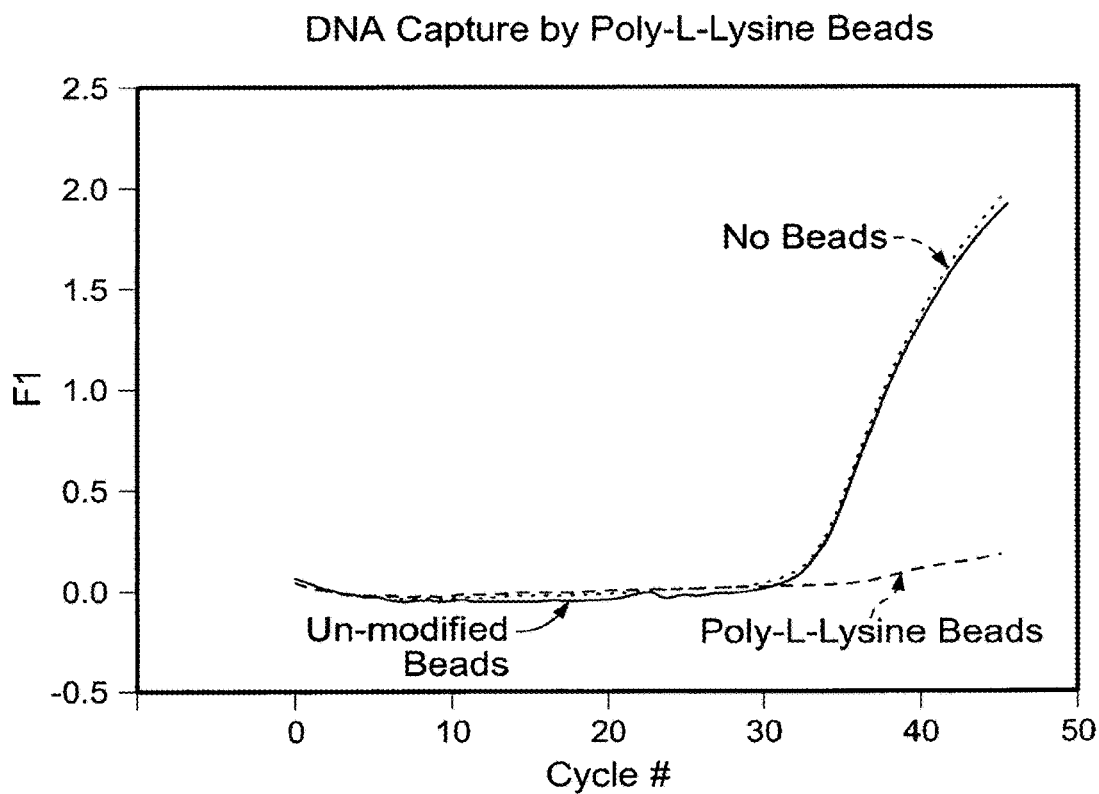


FIG. 22

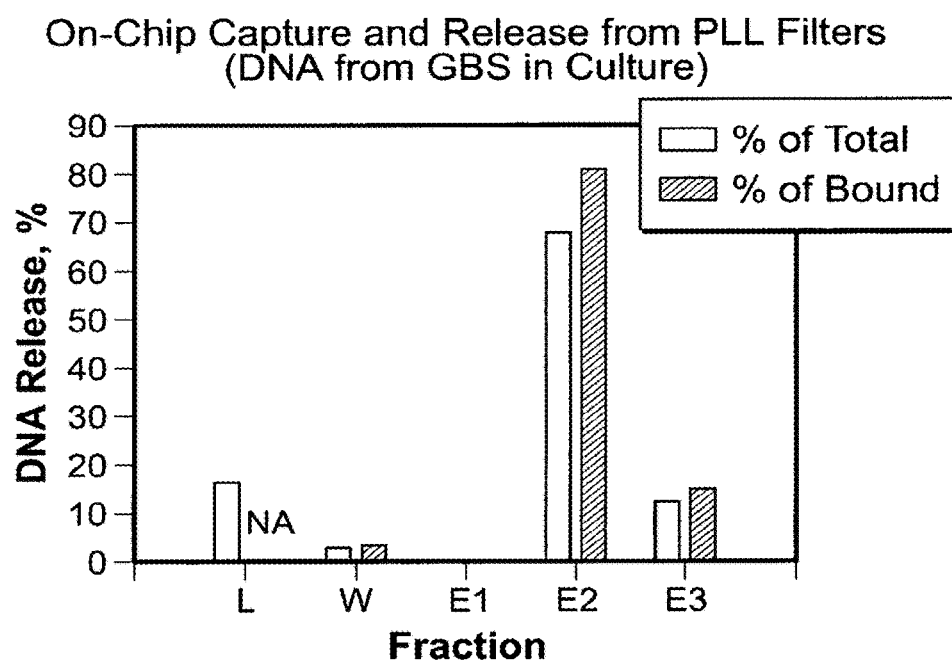


FIG. 23

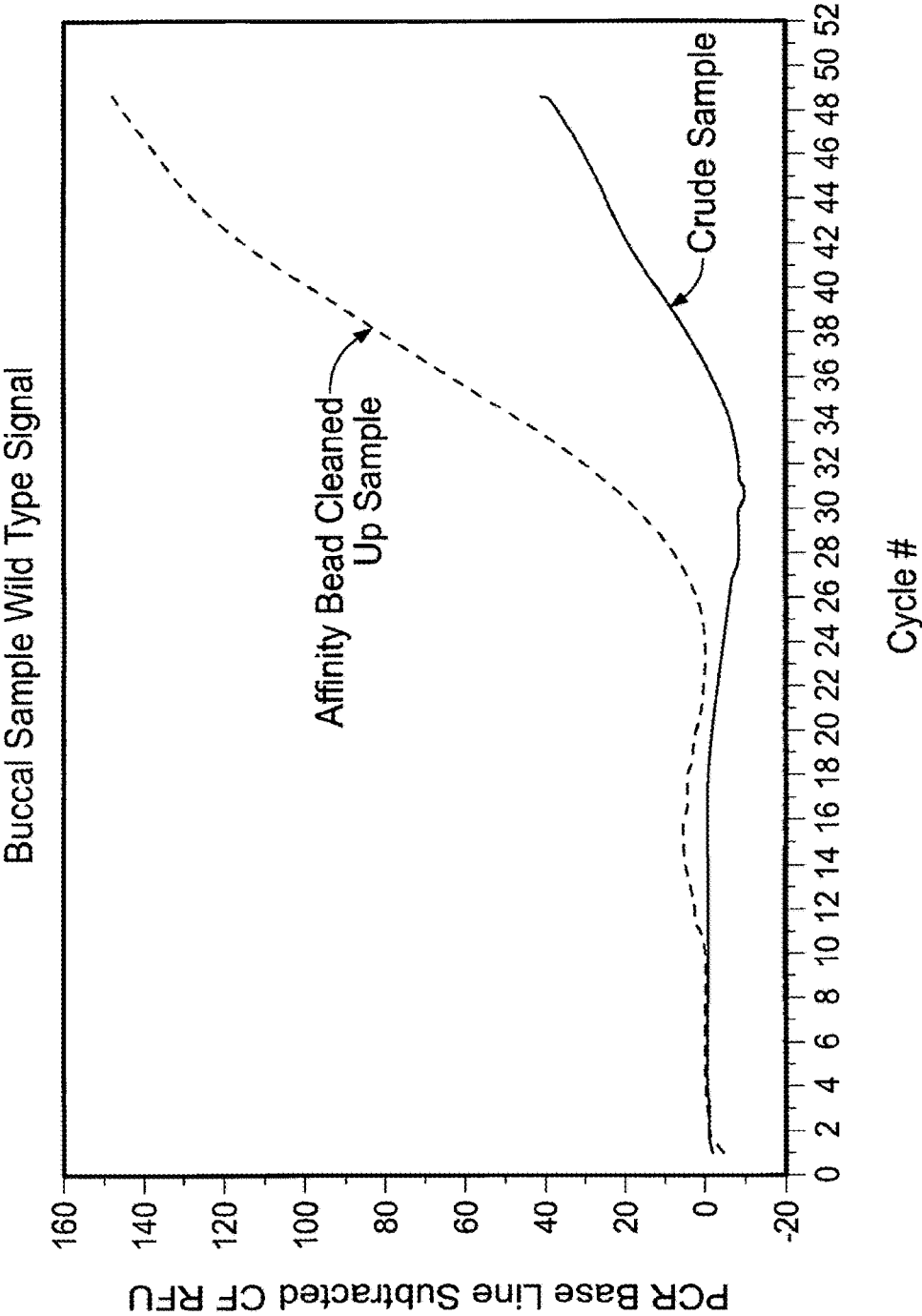


FIG. 24

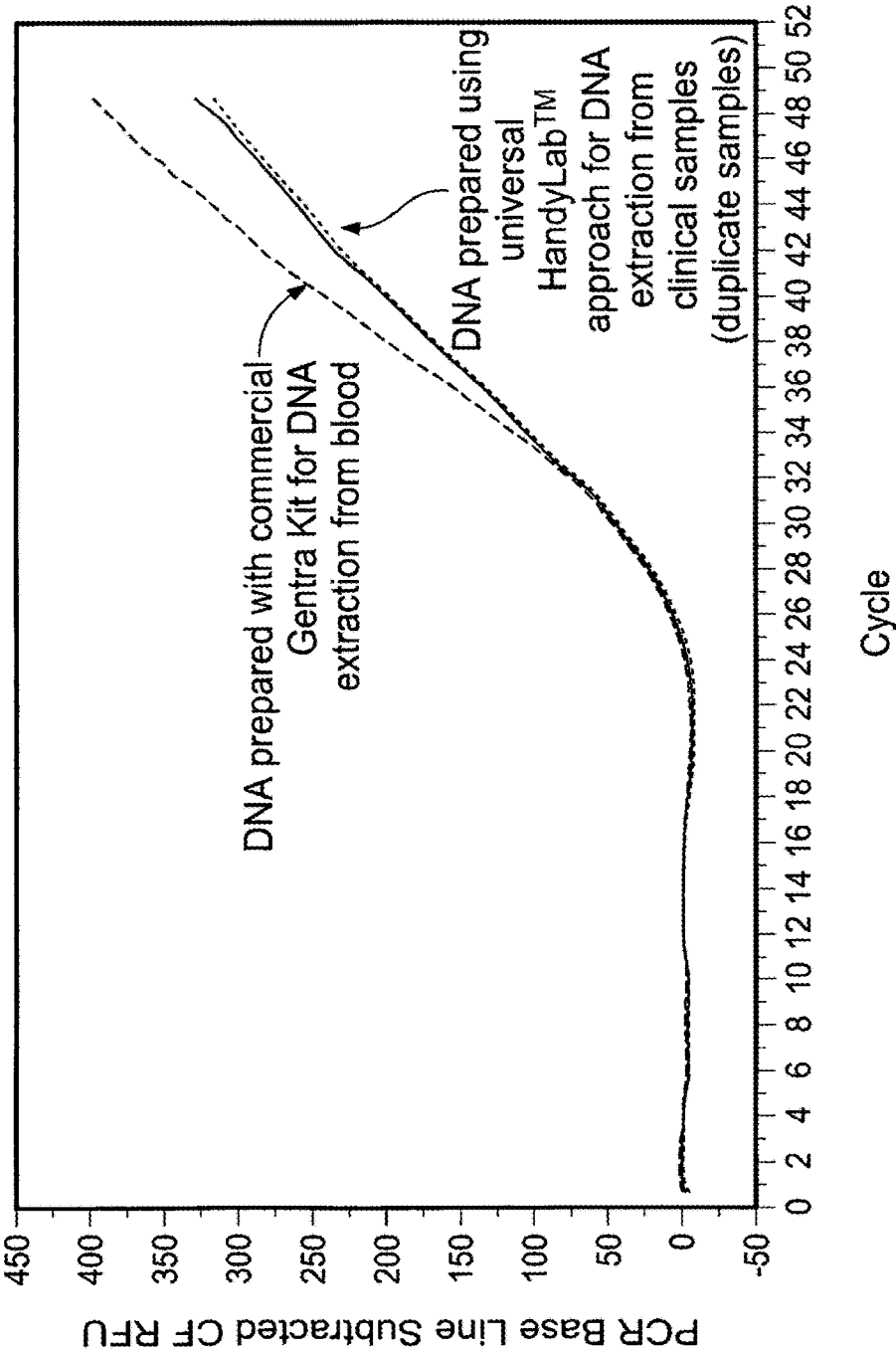


FIG. 25

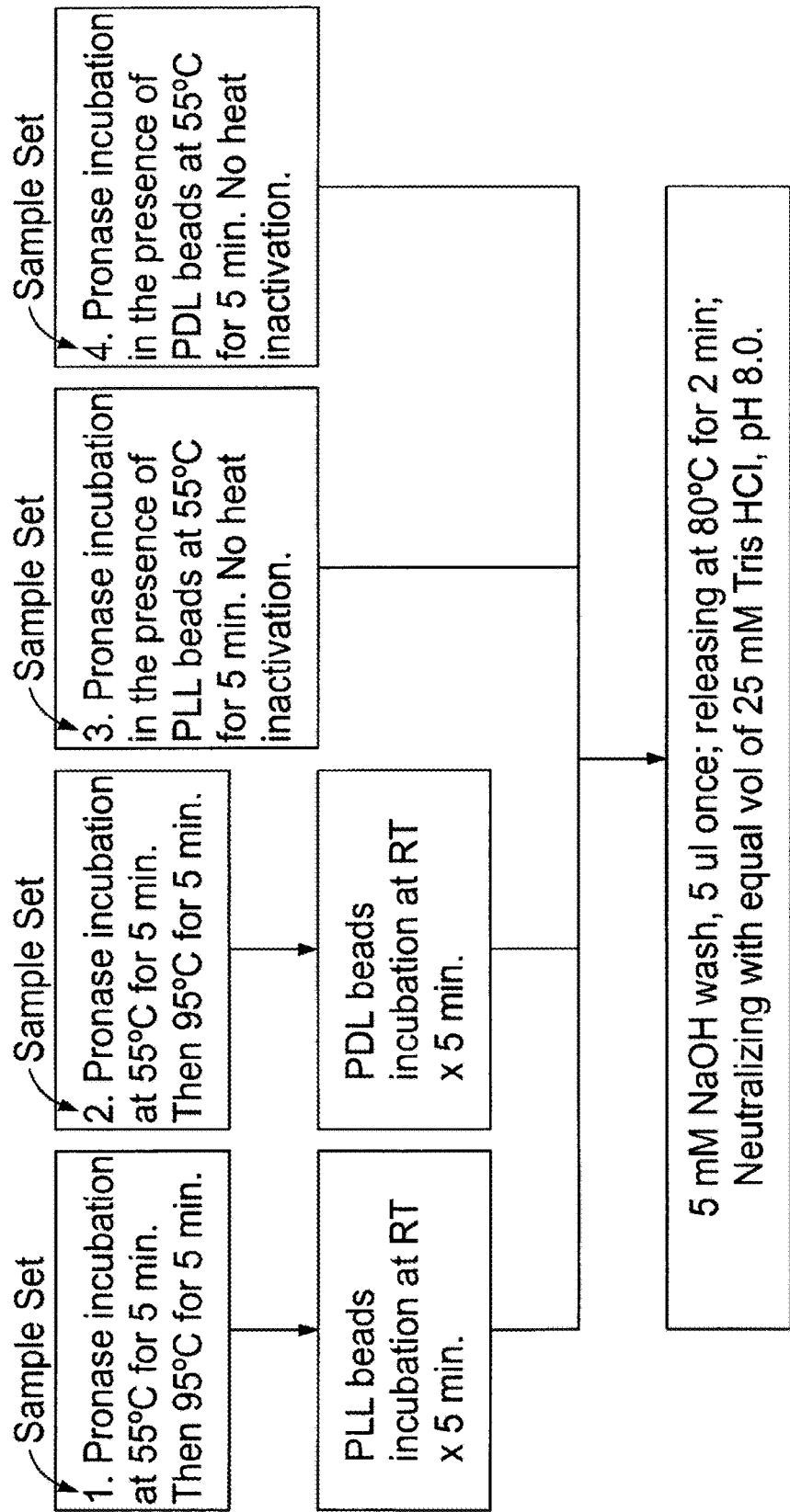


FIG. 26A

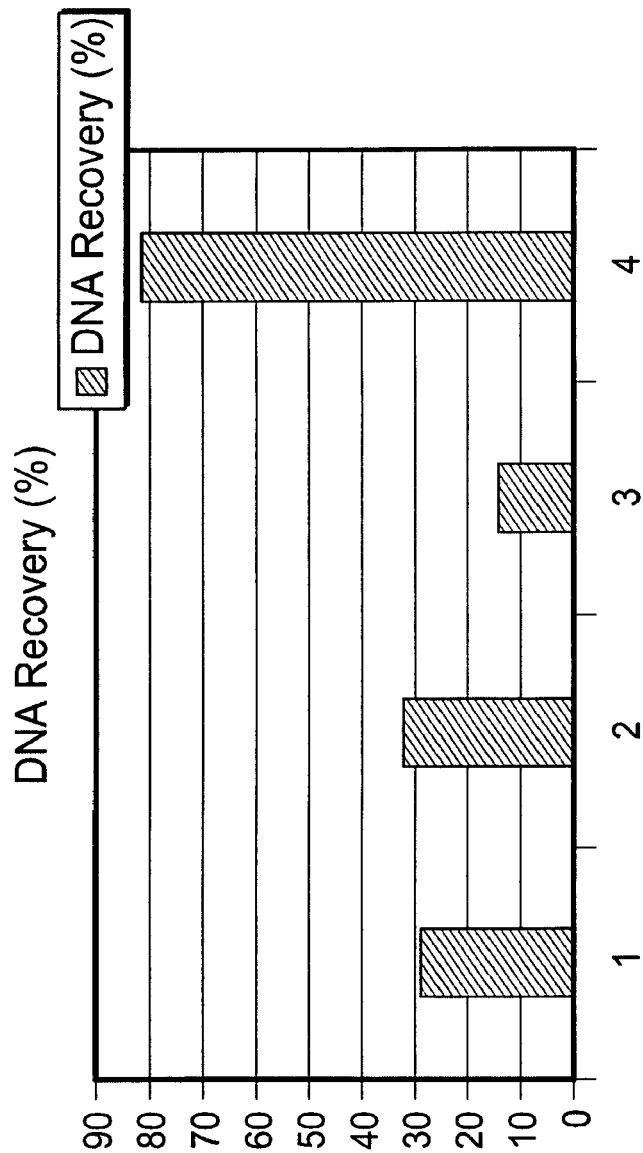


FIG. 26B

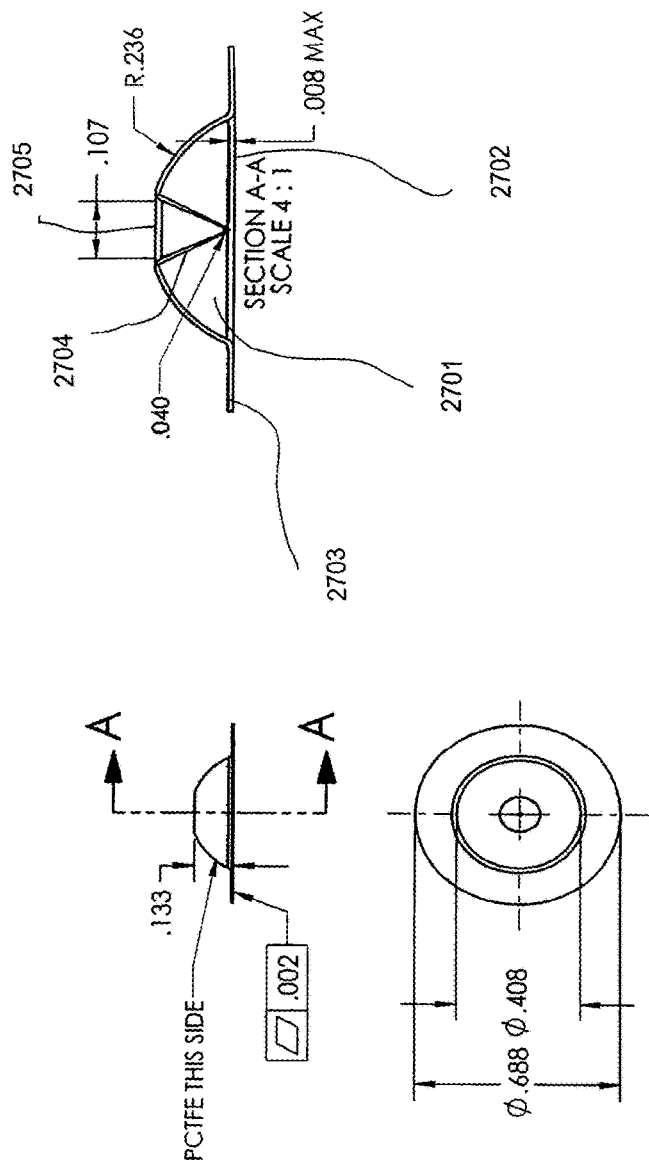


FIG. 27A

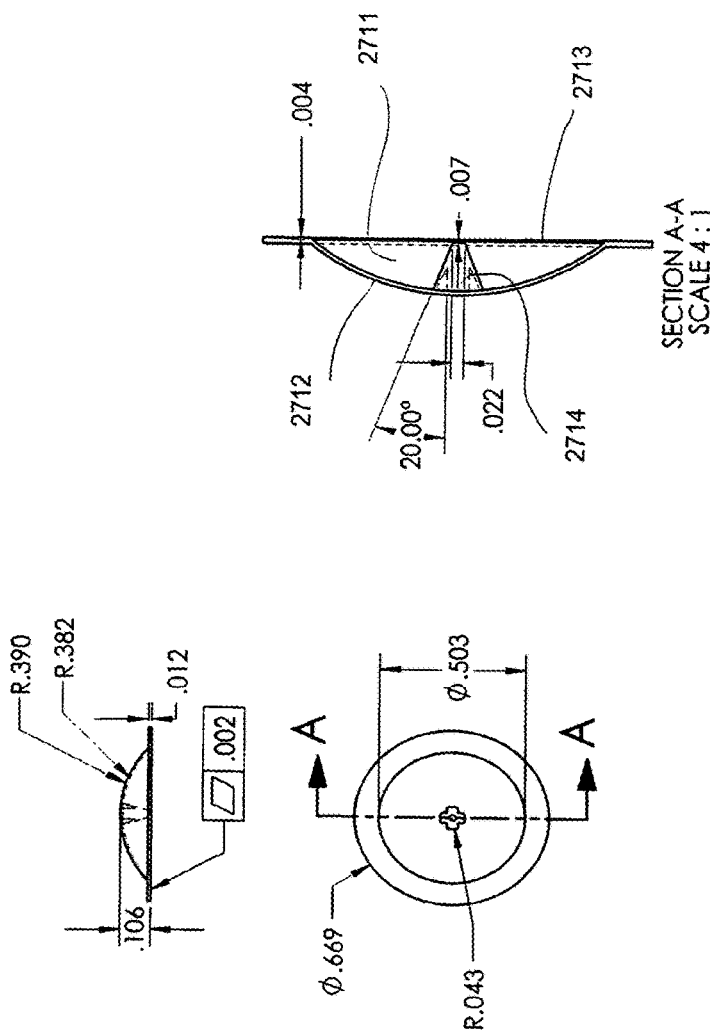


FIG. 27B

US 10,443,088 B1

1

METHOD FOR PROCESSING POLYNUCLEOTIDE-CONTAINING SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 14/506,471, filed Oct. 3, 2014 and scheduled to issue as U.S. Pat. No. 10,364,456 on Jul. 30, 2019, which is a continuation of U.S. patent application Ser. No. 11/281,247, filed Nov. 16, 2005 and issued as U.S. Pat. No. 8,852,862 on Oct. 7, 2014, which is a continuation-in-part of International Application No. PCT/US2005/015345, filed May 3, 2005, which claims the benefit of priority of U.S. Provisional Application No. 60/567,174, filed May 3, 2004, and U.S. Provisional Application No. 60/645,784, filed Jan. 21, 2005. Each of these applications is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods for processing polynucleotide-containing samples as well as to related systems.

BACKGROUND

The analysis of a biological sample often includes detecting one or more polynucleotides present in the sample. One example of detection is qualitative detection, which relates, for example, to the determination of the presence of the polynucleotide and/or the determination of information related to, for example, the type, size, presence or absence of mutations, and/or the sequence of the polynucleotide. Another example of detection is quantitative detection, which relates, for example, to the determination of the amount of polynucleotide present. Detection may include both qualitative and quantitative aspects.

Detecting polynucleotides often involves the use of an enzyme. For example, some detection methods include polynucleotide amplification by polymerase chain reaction (PCR) or a related amplification technique. Other detection methods that do not amplify the polynucleotide to be detected also make use of enzymes. However, the functioning of enzymes used in such techniques may be inhibited by the presence of inhibitors present along with the polynucleotide to be detected. The inhibitors may interfere with, for example, the efficiency and/or specificity of the enzymes.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a method and related systems for processing one or more polynucleotides (e.g., to concentrate the polynucleotide(s) and/or to separate the polynucleotide(s) from inhibitor compounds (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucosal compounds, DNA binding proteins, or a saccharide) that might inhibit detection and/or amplification of the polynucleotides).

In some embodiments, the method includes contacting the polynucleotides and a relatively immobilized compound that preferentially associates with (e.g., retains) the polynucleotides as opposed to inhibitors. An exemplary compound is a poly-cationic polyamide (e.g., poly-L-lysine and/or poly-D-lysine), or polyethyleneimine (PEI), which may be bound to a surface (e.g., a surface of one or more particles). The compound retains the polynucleotides so that the polynucle-

2

otides and inhibitors may be separated, such as by washing the surface with the compound and associated polynucleotides. Upon separation, the association between the polynucleotide and compound may be disrupted to release (e.g., separate) the polynucleotides from the compound and surface.

In some embodiments, the surface (e.g., a surface of one or more particles) is modified with a poly-cationic substance such as a polyamide or PEI, which may be covalently bound to the surface. The poly-cationic polyamide may include at least one of poly-L-lysine and poly-D-lysine. In some embodiments, the poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) have an average molecular weight of at least about 7500 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have an average molecular weight of less than about 35,000 Da (e.g., an average molecular weight of less than about 30000 Da (e.g., an average molecular weight of about 25,000 Da)). The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of at least about 15,000 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of less than about 25,000 Da (e.g., a median molecular weight of less than about 20,000 Da (e.g., a median molecular weight of about 20,000 Da). If the polycationic material is PEI, its molecular weight is preferably in the range 600-800 Daltons.

Another aspect of the invention relates to a sample preparation device including a surface including a polycationic polyamide or PEI bound thereto and a sample introduction passage in communication with the surface for contacting the surface with a fluidic sample.

In some embodiments, the device includes a heat source configured to heat an aqueous liquid in contact with the surface to at least about 65° C.

In some embodiments, the device includes a reservoir of liquid having a pH of at least about 10 (e.g., about 10.5 or more). The device is configured to contact the surface with the liquid (e.g., by actuating a pressure source to move the liquid).

In some embodiments, the surface comprises surfaces of a plurality of particles.

In some embodiments, the poly-cationic polyamide includes poly-L-lysine and/or poly-D-lysine.

Another aspect of the invention relates to a method for processing a sample including providing a mixture including a liquid and an amount of polynucleotide, contacting a retention member with the mixture. The retention member may be configured to preferentially retain polynucleotides as compared to polymerase chain reaction inhibitors. Substantially all of the liquid in the mixture is removed from the retention member. The polynucleotides are released from the retention member. The polynucleotide may have a size of less than about 7.5 Mbp.

The liquid may be a first liquid and removing substantially all of the liquid from the retention member may include contacting the retention member with a second liquid.

Contacting the retention member with a second liquid can include actuating a thermally actuated pressure source to apply a pressure to the second liquid. Contacting the retention member with a second liquid can include opening a thermally actuated valve to place the second liquid in fluid communication with the retention member.

US 10,443,088 B1

3

The second liquid may have a volume of less than about 50 microliters.

The retention member may include a surface having a compound configured to bind polynucleotides preferentially to polymerase chain reaction inhibitors (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucousol compounds, DNA binding proteins, or a saccharide).

The surface may include a poly-lysine (e.g., poly-L-lysine and/or poly-D-lysine) or PEI.

The second liquid may include a detergent (e.g., SDS).

Releasing may include heating the retention member to a temperature of at least about 50° C. (e.g., at about 65° C.). The temperature may be insufficient to boil the liquid in the presence of the retention member during heating. The temperature may be 100° C. or less (e.g., less than 100° C., about 97° C. or less). The temperature may be maintained for less than about 10 minutes (e.g., for less than about 5 minutes, for less than about 3 minutes).

The releasing may be performed without centrifugation of the retention member.

In certain embodiments, PCR inhibitors are rapidly removed from clinical samples to create a PCR-ready sample. The method may comprise the preparation of a polynucleotide-containing sample that is substantially free of inhibitors. The samples may be prepared from, e.g., crude lysates resulting from thermal, chemical, ultrasonic, mechanical, electrostatic, and other lysing techniques. The samples may be prepared without centrifugation. The samples may be prepared using microfluidic devices or on a larger scale.

Another aspect of the invention relates to a retention member, e.g., a plurality of particles such as beads, comprising bound PEI, or poly-lysine, e.g., poly-L-lysine, and related methods and systems. The retention member preferentially binds polynucleotides, e.g., DNA, as compared to inhibitors. The retention member may be used to prepare polynucleotides samples for further processing, such as amplification by polymerase chain reaction.

In certain embodiments, more than 90% of a polynucleotide present in a sample may be bound to the retention member, released, and recovered.

In certain embodiments, a polynucleotide may be bound to the retention member, released, and recovered, in less than about 10 minutes (e.g., less than about 7.5 minutes, less than about 5 minutes, or less than about 3 minutes).

A polynucleotide may be bound to a retention member, released, and recovered without subjecting the polynucleotide, retention member, and/or inhibitors to centrifugation.

Separating the polynucleotides and inhibitors generally excludes subjecting the polynucleotides, inhibitors, processing region, and/or retention member to sedimentation (e.g., centrifugation).

Another aspect of the invention relates to a microfluidic device including a channel, a first mass of a thermally responsive substance (TRS) disposed on a first side of the channel, a second mass of a TRS disposed on a second side of the channel opposite the first side of the channel, a gas pressure source associated with the first mass of the TRS. Actuation of the gas pressure source drives the first mass of the TRS into the second mass of the TRS and obstructs the channel.

The microfluidic device can include a second gas pressure source associated with the second mass of the TRS. Actuation of the second gas pressure source drives the second mass of TRS into the first mass of TRS.

At least one (e.g., both) of the first and second masses of TRS may be a wax.

4

Another aspect of the invention relates to a method for obstructing a channel of a microfluidic device. A mass of a TRS is heated and driven across the channel (e.g., by gas pressure) into a second mass of TRS. The second mass of TRS may also be driven (e.g., by gas pressure) toward the first mass of TRS.

Another aspect of the invention relates to an actuator for a microfluidic device. The actuator includes a channel, a chamber connected to the channel, at least one reservoir of encapsulated liquid disposed in the chamber, and a gas surrounding the reservoir within the chamber. Heating the chamber expands the reservoir of encapsulated liquid and pressurizes the gas. Typically the liquid has a boiling point of about 90° C. or less. The liquid may be a hydrocarbon having about 10 carbon atoms or fewer.

The liquid may be encapsulated by a polymer.

The actuator may include multiple reservoirs of encapsulated liquid disposed in the chamber.

The multiple reservoirs may be dispersed within a solid (e.g., a wax).

The multiple reservoirs may be disposed within a flexible enclosure (e.g., a flexible sack).

Another aspect of the invention relates to a method including pressurizing a gas within a chamber of a microfluidic to create a gas pressure sufficient to move a liquid within a channel of the microfluidic device. Pressurizing the gas typically expanding at least one reservoir of encapsulated liquid disposed within the chamber.

Expanding the at least one reservoir can include heating the chamber.

Pressurizing the gas can include expanding multiple reservoirs of encapsulated liquid.

Another aspect of the invention relates to a method for combining (e.g., mixing) first and second liquids and related devices. The device includes a mass of a temperature responsive substance (TRS) that separates first and second channels of the device. The device is configured to move a first liquid along the first channel so that a portion (e.g., a medial portion) of the first liquid is adjacent the TRS and to move a second liquid along the second channel so that a portion (e.g., a medial portion) of second liquid is adjacent the TRS. A heat source is actuated to move the TRS (e.g., by melting, dispersing, fragmenting). The medial portions of the first and second liquids typically combine without being separated by a gas interface. Typically, only a subset of the first liquid and a subset of the second liquid are combined. The liquids mix upon being moved along a mixing channel.

Another aspect of the invention relates to a lyophilized reagent particle and a method of making the particle.

In some embodiments, the lyophilized particles include multiple smaller particles each having a plurality of ligands that preferentially associate with polynucleotides as compared to PCR inhibitors. The lyophilized particles can also (or alternatively) include lysing reagents (e.g., enzymes) configured to lyse cells to release polynucleotides. The lyophilized particles can also (or alternatively) include enzymes (e.g., proteases) that degrade proteins.

Cells can be lysed by combining a solution of the cells with the lyophilized particles to reconstitute the particles. The reconstituted lysing reagents lyse the cells. The polynucleotides associate with ligands of the smaller particles. During lysis, the solution may be heated (e.g., radiatively using a lamp (e.g., a heat lamp)).

In some embodiments, lyophilized particles include reagents (e.g., primers, control plasmids, polymerase enzymes) for performing PCR.

US 10,443,088 B1

5

A method for making lyophilized particles includes forming a solution of reagents of the particle and a cryoprotectant (e.g., a sugar or poly-alcohol). The solution is deposited dropwise on a chilled hydrophobic surface (e.g., a diamond film or polytetrafluoroethylene surface), without contacting a cooling agent such as liquid nitrogen. The particles freeze and are subjected to reduced pressure (typically while still frozen) for a time sufficient to remove (e.g., sublime) the solvent. The lyophilized particles may have a diameter of about 5 mm or less (e.g., about 2.5 mm or less, about 1.75 mm or less).

Another aspect of the invention relates to a liquid reservoir capable of holding a liquid (e.g., a solvent, a buffer, a reagent, or combination thereof). In general, the reservoir can have one or more of the following features.

The reservoir can include a wall that can be manipulated (e.g., pressed or depressed) to decrease a volume within the reservoir. For example, the reservoir can include a piercing member (e.g., a needle-like or otherwise pointed or sharp member) that ruptures another portion of the reservoir (e.g., a portion of the wall) to release liquid. The piercing member can be internal to the reservoir such that the piercing member ruptures the wall from an inner surface of the reservoir (e.g., wall) outwards.

In general, the wall resists passage of liquid or vapor therethrough. In some embodiments, the wall lacks stretchiness. The wall may be flexible. The wall may be, e.g., a metallic layer, e.g., a foil layer, a polymer, or a laminate including a combination thereof.

The wall may be formed by vacuum formation (e.g., applying a vacuum and heat to a layer of material to draw the layer against a molding surface). The molding surface may be concave such that the wall is provided with a generally convex surface.

Exemplary liquids held by the reservoir include water and aqueous solutions including one or more salts (e.g., magnesium chloride, sodium chloride, Tris buffer, or combination thereof). The reservoir can retain the liquid (e.g., without substantial evaporation thereof) for a period of time (e.g., at least 6 months or at least a year). In some embodiments, less than 10% (e.g., less than about 5%) by weight of the liquid evaporates over a year.

The piercing member may be an integral part of a wall of the reservoir. For example, the reservoir can include a wall having an internal projection, which may be in contact with liquid in the reservoir. The reservoir also includes a second wall opposite the piercing member. During actuation, the piercing member is driven through the second wall (e.g., from the inside out) to release liquid.

In some embodiments, a maximum amount of liquid retained by a reservoir is less than about 1 ml. For example, a reservoir may hold about 500 microliters or less (e.g., 300 microliters or less). Generally, a reservoir holds at least about 25 microliters (e.g., at least about 50 microliters). The reservoir can introduce within about 10% of the intended amount of liquid (e.g., 50 ± 5 μ l).

The reservoir can deliver a predetermined amount of liquid that is substantially air-free (e.g., substantially gas-free). Upon introduction of the liquid, the substantially air and/or gas free liquid produces few or no bubbles large enough to obstruct movement of the liquid within the microfluidic device. Use of a piercing member internal to the reservoir can enhance an ability of the reservoir to deliver substantially air and/or gas free liquids.

In some embodiments, the reservoir can be actuated to release liquid by pressing (e.g., by one's finger or thumb or by mechanical pressure actuation). The pressure may be

6

applied directly to a wall of the reservoir or to a plunger having a piercing member. In embodiments, minimal pressure is required to actuate the reservoir. An automated system can be used to actuate (e.g., press upon) a plurality of reservoirs simultaneously or in sequence.

In some embodiments, the reservoir does not include a piercing member. Instead, internal pressure generated within the reservoir ruptures a wall of the reservoir allowing liquid to enter the microfluidic device.

Upon actuating a reservoir to introduce liquid into the microfluidic device, liquid generally does not withdraw back into the reservoir. For example, upon actuation, the volume of the reservoir may decrease to some minimum but generally does not increase so as to withdraw liquid back into the reservoir. For example, the reservoir may stay collapsed upon actuation. In such embodiments, the flexible wall may be flexible but lack hysteresis or stretchiness. Alternatively or in combination, the reservoir may draw in air from a vent without withdrawing any of the liquid.

Actuation of the reservoir may include driving a piercing member through a wall of the reservoir.

The reservoir preserves the reactivity and composition of reagents therein (e.g., the chemicals within the reservoir may exhibit little or no change in reactivity over 6 months or a year).

The flexible wall of the reservoir can limit or prevent leaching of chemicals therethrough. The reservoir can be assembled independently of a microfluidic device and then secured to the microfluidic device.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of a microfluidic device.

FIG. 2 is a cross-sectional view of a processing region for retaining polynucleotides and/or separating polynucleotides from inhibitors.

FIG. 3 is a cross-sectional view of an actuator.

FIG. 4 is a perspective view of a microfluidic device.

FIG. 5 is a side cross-sectional view of the microfluidic device of FIG. 4.

FIGS. 6A and 6B, taken together, illustrate a perspective view of a microfluidic network of the microfluidic device of FIG. 4.

FIG. 7 illustrates an array of heat sources for operating components of the microfluidic device of FIG. 4.

FIGS. 8 and 9 illustrate a valve in the open and closed states respectively.

FIG. 10A-10D illustrate a mixing gate of the microfluidic network of FIGS. 6A and 6B and adjacent regions of the network.

FIGS. 11A-11C illustrate a reservoir with actuation mechanism.

FIGS. 12A-12C illustrate a reservoir with actuation mechanism.

FIG. 13 illustrates a reservoir with actuation mechanism.

FIGS. 14A-14B illustrate a reservoir with actuation mechanism.

FIGS. 15A-15B illustrate a reservoir with actuation mechanism.

FIG. 16 illustrates a reservoir with actuation mechanism.

FIG. 17 illustrates a reservoir with actuation mechanism.

FIG. 18 illustrates a device for separating polynucleotides and inhibitors.

FIG. 19 illustrates the device of FIG. 18 and a device for operation thereof.

FIG. 20 illustrates a microfluidic device.

US 10,443,088 B1

7

FIG. 21 is a cross-section of the microfluidic device of FIG. 20 taken along 5.

FIG. 22 illustrates the retention of herring sperm DNA.

FIG. 23 illustrates the retention and release of DNA from group B streptococci;

FIG. 24 illustrates the PCR response of a sample from which inhibitors had been removed and of a sample from which inhibitors had not been removed.

FIG. 25 illustrates the PCR response of a sample prepared in accord with the invention and a sample prepared using a commercial DNA extraction method.

FIG. 26A illustrates a flow chart showing steps performed during a method for separating polynucleotides and inhibitors.

FIG. 26B illustrates DNA from samples subjected to the method of FIG. 26A.

FIGS. 27A and 27B show, respectively, two embodiments of a reservoir with a piercing member.

DETAILED DESCRIPTION OF THE INVENTION

Analysis of biological samples often includes determining whether one or more polynucleotides (e.g., a DNA, RNA, mRNA, or rRNA) is present in the sample. For example, one may analyze a sample to determine whether a polynucleotide indicative of the presence of a particular pathogen is present. Typically, biological samples are complex mixtures. For example, a sample may be provided as a blood sample, a tissue sample (e.g., a swab of, for example, nasal, buccal, anal, or vaginal tissue), a biopsy aspirate, a lysate, as fungi, or as bacteria. Polynucleotides to be determined may be contained within particles (e.g., cells (e.g., white blood cells and/or red blood cells), tissue fragments, bacteria (e.g., gram positive bacteria and/or gram negative bacteria), fungi, spores). One or more liquids (e.g., water, a buffer, blood, blood plasma, saliva, urine, spinal fluid, or organic solvent) is typically part of the sample and/or is added to the sample during a processing step.

Methods for analyzing biological samples include providing a biological sample (e.g., a swab), releasing polynucleotides from particles (e.g., bacteria) of the sample, amplifying one or more of the released polynucleotides (e.g., by polymerase chain reaction (PCR)), and determining the presence (or absence) of the amplified polynucleotide(s) (e.g., by fluorescence detection). Biological samples, however, typically include inhibitors (e.g., mucosal compounds, hemoglobin, faecal compounds, and DNA binding proteins) that can inhibit determining the presence of polynucleotides in the sample. For example, such inhibitors can reduce the amplification efficiency of polynucleotides by PCR and other enzymatic techniques for determining the presence of polynucleotides. If the concentration of inhibitors is not reduced relative to the polynucleotides to be determined, the analysis can produce false negative results.

We describe methods and related systems for processing biological samples (e.g., samples having one or more polynucleotides to be determined). Typically, the methods and systems reduce the concentration of inhibitors relative to the concentration of polynucleotides to be determined.

Referring to FIG. 1, a microfluidic device 200 includes first, second, and third layers 205, 207, and 209 that define a microfluidic network 201 having various components configured to process a sample including one or more polynucleotides to be determined. Device 200 typically processes the sample by increasing the concentration of a polynucleotide to be determined and/or by reducing the

8

concentration of inhibitors relative to the concentration of polynucleotide to be determined.

We now discuss the arrangement of components of network 201.

Network 201 includes an inlet 202 by which sample material can be introduced to the network and an output 236 by which a processed sample can be removed (e.g., expelled by or extracted from) network 201. A channel 204 extends between inlet 202 and a junction 255. A valve 206 is positioned along channel 204. A reservoir channel 240 extends between junction 255 and an actuator 244. Gates 242 and 246 are positioned along channel 240. A channel 257 extends between junction 255 and a junction 259. A valve 208 is positioned along channel 257. A reservoir channel 246 extends between junction 259 and an actuator 248. Gates 250 and 252 are positioned along channel 246. A channel 261 extends between junction 259 and a junction 263. A valve 210 and a hydrophobic vent 212 are positioned along channel 261. A channel 256 extends between junction 263 and an actuator 254. A gate 258 is positioned along channel 256.

A channel 214 extends between junction 263 and a processing chamber 220, which has an inlet 265 and an outlet 267. A channel 228 extends between processing chamber outlet 267 and a waste reservoir 232. A valve 234 is positioned along channel 228. A channel 230 extends between processing chamber outlet 267 and output 236.

We turn now to particular components of network 201.

Referring also to FIG. 2, processing chamber 220 includes a plurality of particles (e.g., beads, microspheres) 218 configured to retain polynucleotides of the sample under a first set of conditions (e.g., a first temperature and/or first pH) and to release the polynucleotides under a second set of conditions (e.g., a second, higher temperature and/or a second, more basic pH). Typically, the polynucleotides are retained preferentially as compared to inhibitors that may be present in the sample. Particles 218 are configured as a retention member 216 (e.g., a column) through which sample material (e.g., polynucleotides) must pass when moving between the inlet 265 and outlet 267 of processing region 220.

A filter 219 prevents particles 218 from passing downstream of processing region 220. A channel 287 connects filter 219 with outlet 267. Filter 219 has a surface area within processing region 220 that is larger than the cross-sectional area of inlet 265. For example, in some embodiments, the ratio of the surface area of filter 219 within processing region 220 to the cross-sectional area of inlet 265 (which cross-sectional area is typically about the same as the cross-sectional area of channel 214) is at least about 5 (e.g., at least about 10, at least about 20, at least about 30). In some embodiments, the surface area of filter 219 within processing region 220 is at least about 1 mm² (e.g., at least about 2 mm², at least about 3 mm²). In some embodiments, the cross-sectional area of inlet 265 and/or channel 214 is about 0.25 mm² or less (e.g., about 0.2 mm² or less, about 0.15 mm² or less, about 0.1 mm² or less). The larger surface area presented by filter 219 to material flowing through processing region 220 helps prevent clogging of the processing region while avoiding significant increases in the void volume (discussed below) of the processing region.

Particles 218 are modified with at least one ligand that retains polynucleotides (e.g., preferentially as compared to inhibitors). Typically, the ligands retain polynucleotides from liquids having a pH about 9.5 or less (e.g., about 9.0 or less, about 8.75 or less, about 8.5 or less). As a sample solution moves through processing region 220, polynucle-

US 10,443,088 B1

9

otides are retained while the liquid and other solution components (e.g., inhibitors) are less retained (e.g., not retained) and exit the processing region. In general, the ligands release polynucleotides when the pH is about 10 or greater (e.g., about 10.5 or greater, about 11.0 or greater, about 11.4 or greater). Consequently, polynucleotides can be released from the ligand modified particles into the surrounding liquid.

Exemplary ligands include, for example, polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine) and PEI. Other ligands include, for example, intercalators, poly-intercalators, minor groove binders polyamines (e.g., spermidine), homopolymers and copolymers comprising a plurality of amino acids, and combinations thereof. In some embodiments, the ligands have an average molecular weight of at least about 5000 Da (e.g., at least about 7500 Da, of at least about 15000 Da). In some embodiments, the ligands have an average molecular weight of about 50000 Da or less (e.g., about 35000, or less, about 27500 Da or less). In some embodiments, the ligand is a poly-lysine ligand attached to the particle surface by an amide bond.

In certain embodiments, the ligands are resistant to enzymatic degradation, such as degradation by protease enzymes (e.g., mixtures of endo- and exo-proteases such as pronase) that cleave peptide bonds. Exemplary protease resistant ligands include, for example, poly-D-lysine and other ligands that are enantiomers of ligands susceptible to enzymatic attack.

Particles **218** are typically formed of a material to which the ligands can be associated. Exemplary materials from which particles **218** can be formed include polymeric materials that can be modified to attach a ligand. Typical polymeric materials provide or can be modified to provide carboxylic groups and/or amino groups available to attach ligands. Exemplary polymeric materials include, for example, polystyrene, latex polymers (e.g., polycarboxylate coated latex), polyacrylamide, polyethylene oxide, and derivatives thereof. Polymeric materials that can be used to form particles **218** are described in U.S. Pat. No. 6,235,313 to Mathiowitz et al., which patent is incorporated herein by reference. Other materials include glass, silica, agarose, and amino-propyl-tri-ethoxy-silane (APES) modified materials.

Exemplary particles that can be modified with suitable ligands include carboxylate particles (e.g., carboxylate modified magnetic beads (Sera-Mag Magnetic Carboxylate modified beads, Part #3008050250, Seradyn) and Polybead carboxylate modified microspheres available from Polyscience, catalog no. 09850). In some embodiments, the ligands include poly-D-lysine and the beads comprise a polymer (e.g., polycarboxylate coated latex). In other embodiments, the ligands include PEI.

In general, the ratio of mass of particles to the mass of polynucleotides retained by the particles is no more than about 25 or more (e.g., no more than about 20, no more than about 10). For example, in some embodiments, about 1 gram of particles retains about 100 milligrams of polynucleotides.

Typically, the total volume of processing region **220** (including particles **218**) between inlet **265** and filter **219** is about 15 microliters or less (e.g., about 10 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. In some embodiments, particles **218** occupy at least about 10 percent (e.g., at least about 15 percent) of the total volume of processing region **220**. In some embodiments, particles **218** occupy about 75 percent or less (e.g.,

10

about 50 percent or less, about 35 percent or less) of the total volume of processing chamber **220**.

In some embodiments, the volume of processing region **220** that is free to be occupied by liquid (e.g., the void volume of processing region **220** including interstices between particles **218**) is about equal to the total volume minus the volume occupied by the particles. Typically, the void volume of processing region **220** is about 10 microliters or less (e.g., about 7.5 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In some embodiments, the void volume is about 50 nanoliters or more (e.g., about 100 nanoliters or more, about 250 nanoliters or more). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. For example, in an exemplary embodiment, the total volume of the processing region is about 2.3 microliters, the volume occupied by particles is about 0.3 microliters, and the volume free to be occupied by liquid (void volume) is about 2 microliters.

Particles **218** typically have an average diameter of about 20 microns or less (e.g., about 15 microns or less, about 10 microns or less). In some embodiments, particles **218** have an average diameter of at least about 4 microns (e.g., at least about 6 microns, at least about 8 microns).

In some embodiments, a volume of channel **287** between filter **219** and outlet **267** is substantially smaller than the void volume of processing region **220**. For example, in some embodiments, the volume of channel **287** between filter **219** and outlet **267** is about 35% or less (e.g., about 25% or less, about 20% or less) of the void volume. In an exemplary embodiment, the volume of channel **287** between filter **219** and outlet **267** is about 500 nanoliters.

The particle density is typically at least about 10^8 particles per milliliter (e.g., about 10^9 particles per milliliter). For example, a processing region with a total volume of about 1 microliter may include about 103 beads.

Filter **219** typically has pores with a width smaller than the diameter of particles **218**. In an exemplary embodiment, filter **219** has pores having an average width of about 8 microns and particles **218** have an average diameter of about 10 microns.

In some embodiments, at least some (e.g., all) of the particles are magnetic. In alternative embodiments, few (e.g., none) of the particles are magnetic.

In some embodiments, at least some (e.g., all) the particles are solid. In some embodiments, at least some (e.g., all) the particles are porous (e.g., the particles may have channels extending at least partially within them).

We continue discussing components of network **201**.

Channels of microfluidic network **201** typically have at least one sub-millimeter cross-sectional dimension. For example, channels of network **201** may have a width and/or a depth of about 1 mm or less (e.g., about 750 microns or less, about 500 microns, or less, about 250 microns or less).

A valve is a component that has a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Upon actuation, the valve transitions to a closed state that prevents material from passing along the channel from one side of the valve to the other. For example, valve **206** includes a mass **251** of a thermally responsive substance (TRS) that is relatively immobile at a first temperature and more mobile at a second temperature. A chamber **253** is in gaseous communication with mass **251**. Upon heating gas (e.g., air) in chamber **253** and heating mass **251** of TRS to the second temperature, gas pressure within chamber **253**

US 10,443,088 B1

11

moves mass **251** into channel **204** obstructing material from passing therealong. Other valves of network **201** have the same structure and operate in the same fashion as valve **206**.

A mass of TRS can be an essentially solid mass or an agglomeration of smaller particles that cooperate to obstruct the passage. Examples of TRS's include a eutectic alloy (e.g., a solder), wax (e.g., an olefin), polymers, plastics, and combinations thereof. The first and second temperatures are insufficiently high to damage materials, such as polymer layers of device **200**. Generally, the second temperature is less than about 90° C. and the first temperature is less than the second temperature (e.g., about 70° C. or less).

A gate is a component that has a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Upon actuation, the gate transitions to an open state in which material is permitted to pass from one side of the gate (e.g., upstream of the gate) to the other side of the gate (e.g., downstream of the gate). For example, gate **242** includes a mass **271** of TRS positioned to obstruct passage of material between junction **255** and channel **240**. Upon heating mass **271** to the second temperature, the mass changes state (e.g., by melting, by dispersing, by fragmenting, and/or dissolving) to permit passage of material between junction **255** and channel **240**.

The portion of channel **240** between gates **242** and **246** forms a fluid reservoir **279** configured to hold a liquid (e.g., water, an organic liquid, or combination thereof). During storage, gates **242** and **246** limit (e.g., prevent) evaporation of liquid within the fluid reservoir. During operation of device **200**, the liquid of reservoir **279** is typically used as a wash liquid to remove inhibitors from processing region **220** while leaving polynucleotides associated with particles **218**. Typically, the wash liquid is a solution having one or more additional components (e.g., a buffer, chelator, surfactant, a detergent, a base, an acid, or a combination thereof). Exemplary solutions include, for example, a solution of 10-50 mM Tris at pH 8.0, 0.5-2 mM EDTA, and 0.5%-2% SDS, a solution of 10-50 mM Tris at pH 8.0, 0.5 to 2 mM EDTA, and 0.5%-2% Triton X-100.

The portion of channel **246** between gates **250** and **252** form a fluid reservoir **281** configured like reservoir **279** to hold a liquid (e.g., a solution) with limited or no evaporation. During operation of device **200**, the liquid of reservoir **281** is typically used as a release liquid into which polynucleotides that had been retained by particles **218** are released. An exemplary release liquid is an hydroxide solution (e.g., a NaOH solution) having a concentration of, for example, between about 2 mM hydroxide (e.g., about 2 mM NaOH) and about 500 mM hydroxide (e.g., about 500 mM NaOH). In some embodiments, liquid in reservoir **281** is an hydroxide solution having a concentration of about 25 mM or less (e.g., an hydroxide concentration of about 15 mM).

Reservoirs **279**, **281** typically hold at least about 0.375 microliters of liquid (e.g., at least about 0.750 microliters, at least about 1.25 microliters, at least about 2.5 microliters). In some embodiments, reservoirs **279**, **281** hold about 7.5 microliters or less of liquid (e.g., about 5 microliters or less, about 4 microliters or less, about 3 microliters or less).

An actuator is a component that provides a gas pressure that can move material (e.g., sample material and/or reagent material) between one location of network **201** and another location. For example, referring to FIG. 3, actuator **244** includes a chamber **272** having a mass **273** of thermally expansive material (TEM) therein. When heated, the TEM expands decreasing the free volume within chamber **272** and pressurizing the gas (e.g., air) surrounding mass **273** within

12

chamber **272**. Typically, gates **246** and **242** are actuated with actuator **244**. Consequently, the pressurized gas drives liquid in fluid reservoir **279** towards junction **255**. In some embodiments, actuator **244** can generate a pressure differential of more than about 3 psi (e.g., at least about 4 psi, at least about 5 psi) between the actuator and junction **255**.

The TEM includes a plurality of sealed liquid reservoirs (e.g., spheres) **275** dispersed within a carrier **277**. Typically, the liquid is a high vapor pressure liquid (e.g., isobutane and/or isopentane) sealed within a casing (e.g., a polymeric casing formed of monomers such as vinylidene chloride, acrylonitrile and methymethacrylate). Carrier **277** has properties (e.g., flexibility and/or an ability to soften (e.g., melt) at higher temperatures) that permit expansion of the reservoirs **275** without allowing the reservoirs to pass along channel **240**. In some embodiments, carrier **277** is a wax (e.g., an olefin) or a polymer with a suitable glass transition temperature. Typically, the reservoirs make up at least about 25 weight percent (e.g., at least about 35 weight percent, at least about 50 weight percent) of the TEM. In some embodiments, the reservoirs make up about 75 weight percent or less (e.g., about 65 weight percent or less, about 50 weight percent or less) of the TEM. Suitable sealed liquid reservoirs can be obtained from Expancel (Akzo Nobel).

When the TEM is heated (e.g., to a temperature of at least about 50° C. (e.g., to at least about 75° C., at least about 90° C.)), the liquid vaporizes and increases the volume of each sealed reservoir and of mass **273**. Carrier **277** softens allowing mass **273** to expand. Typically, the TEM is heated to a temperature of less than about 150° C. (e.g., about 125° C. or less, about 110° C. or less, about 100° C. or less) during actuation. In some embodiments, the volume of the TEM expands by at least about 5 times (e.g., at least about 10 times, at least about 20 times, at least about 30 times).

A hydrophobic vent (e.g., vent **212**) is a structure that permits gas to exit a channel while limiting (e.g., preventing) liquid from exiting the channel. Typically, hydrophobic vents include a layer of porous hydrophobic material (e.g., a porous filter such as a porous hydrophobic membrane from Osmonics) that defines a wall of the channel. As discussed below, hydrophobic vents can be used to position a microdroplet of sample at a desired location within network **201**.

The hydrophobic vents of the present invention are preferably constructed so that the amount of air that escapes through them is maximized while minimizing the volume of the channel below the vent surface. Accordingly, it is preferable that the vent is constructed so as to have a hydrophobic membrane of large surface area and a shallow cross section of the microchannel below the vent surface.

Hydrophobic vents typically have a length of at least about 2.5 mm (e.g., at least about 5 mm, at least about 7.5 mm) along a channel. The length of the hydrophobic vent is typically at least about 5 times (e.g., at least about 10 times, at least about 20 times) larger than a depth of the channel within the hydrophobic vent. For example, in some embodiments, the channel depth within the hydrophobic vent is about 300 microns or less (e.g., about 250 microns or less, about 200 microns or less, about 150 microns or less).

The depth of the channel within the hydrophobic vent is typically about 75% or less (e.g., about 65% or less, about 60% or less) of than the depth of the channel upstream and downstream of the hydrophobic vent. For example, in some embodiments the channel depth within the hydrophobic vent is about 150 microns and the channel depth upstream and downstream of the hydrophobic vent is about 250 microns.

A width of the channel within the hydrophobic vent is typically at least about 25% wider (e.g., at least about 50%

US 10,443,088 B1

13

wider) than a width of the channel upstream from the vent and downstream from the vent. For example, in an exemplary embodiment, the width of the channel within the hydrophobic vent is about 400 microns and the width of the channel upstream and downstream from the vent is about 250 microns.

Microfluidic device 200 can be fabricated as desired. Typically, layers 205, 207, and 209 are formed of a polymeric material. Components of network 201 are typically formed by molding (e.g., by injection molding) layers 207, 209. Layer 205 is typically a flexible polymeric material (e.g., a laminate) that is secured (e.g., adhesively and/or thermally) to layer 207 to seal components of network 201. Layers 207 and 209 may be secured to one another using adhesive.

In use, device 200 is typically thermally associated with an array of heat sources configured to operate the components (e.g., valves, gates, actuators, and processing region 220) of the device. In some embodiments, the heat sources are operated by an operating system, which operates the device during use. The operating system includes a processor (e.g., a computer) configured to actuate the heat sources according to a desired protocol. Processors configured to operate microfluidic devices are described in U.S. application Ser. No. 09/819,105, filed Mar. 28, 2001, which application is incorporated herein by reference. In other embodiments, the heat sources are integral with the device itself.

Device 200 may be operated as follows. Valves of network 201 are configured in the open state. Gates of network 201 are configured in the closed state. A fluidic sample comprising polynucleotides is introduced to network 201 via inlet 202. For example, sample can be introduced with a syringe having a Luer fitting. The syringe provides pressure to initially move the sample within network 201. Sample passes along channels 204, 257, 261, and 214 to inlet 265 of processing region 220. The sample passes through processing region 220, exits via outlet 267, and passes along channel 228 to waste chamber 232. When the trailing edge (e.g., the upstream liquid-gas interface) of the sample reaches hydrophobic vent 212, pressure provided by the introduction device (e.g., the syringe) is released from network 201 stopping further motion of the sample.

Typically, the amount of sample introduced is about 500 microliters or less (e.g., about 250 microliters or less, about 100 microliters or less, about 50 microliters or less, about 25 microliters or less, about 10 microliters or less). In some embodiments, the amount of sample is about 2 microliters or less (e.g., of about 0.5 microliters or less).

Polynucleotides entering processing region 220 pass through interstices between the particles 218. Polynucleotides of the sample contact retention member 216 and are preferentially retained as compared to liquid of the sample and certain other sample components (e.g., inhibitors). Typically, retention member 220 retains at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region 220. Liquid of the sample and inhibitors present in the sample exit the processing region 220 via outlet 267 and enter waste chamber 232. Processing region 220 is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during introduction of the sample.

Processing continues by washing retention member 216 with liquid of reservoir 279 to separate remaining inhibitors from polynucleotides retained by retention member 216. To wash retention member 216, valve 206 is closed and gates 242, 246 of first reservoir 240 are opened. Actuator 244 is

14

actuated and moves wash liquid within reservoir 279 along channels 257, 261, and 214, through processing region 220, and into waste reservoir 232. The wash liquid moves sample that may have remained within channels 204, 257, 261, and 214 through the processing region and into waste chamber 232. Once the trailing edge of the wash liquid reaches vent 212, the gas pressure generated by actuator 244 is vented and further motion of the liquid is stopped.

The volume of wash liquid moved by actuator 244 through processing region 220 is typically at least about 2 times the void volume of processing region 220 (e.g., at least about 3 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less). Processing region is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during washing. Exemplary wash fluids include liquids discussed with respect to reservoirs 279 and 281.

Processing continues by releasing polynucleotides from retention member 216. Typically, wash liquid from reservoir 279 is replaced with release liquid (e.g., an hydroxide solution) from reservoir 281 before releasing the polynucleotides. Valve 208 is closed and gates 250, 252 are opened. Actuator 248 is actuated thereby moving release liquid within reservoir 281 along channels 261, 214 and into processing region 220 and in contact with retention member 216. When the trailing edge of release liquid from reservoir 281 reaches hydrophobic vent 212, pressure generated by actuator 248 is vented stopping the further motion of the liquid. The volume of liquid moved by actuator 248 through processing region 220 is typically at least about equal to the void volume of the processing region 220 (e.g., at least about 2 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less).

Once retention member 216 with retained polynucleotides has been contacted with liquid from reservoir 281, a releasing step is typically performed. Typically, the releasing step includes heating release liquid present within processing region 216. Generally, the liquid is heated to a temperature insufficient to boil liquid in the presence of the retention member. In some embodiments, the temperature is 100° C. or less (e.g., less than 100° C., about 97° C. or less). In some embodiments, the temperature is about 65° C. or more (e.g., about 75° C. or more, about 80° C. or more, about 90° C. or more). In some embodiments, the temperature maintained for about 1 minute or more (e.g., about 2 minutes or more, about 5 minutes or more, about 10 minutes or more). In some embodiments, the temperature is maintained for about 30 minutes (e.g., about 15 minutes or less, about 10 minutes or less, about 5 minutes or less). In an exemplary embodiment, processing region 220 is heated to between about 65 and 90° C. (e.g., to about 70° C.) for between about 1 and 7 minutes (e.g., for about 2 minutes).

The polynucleotides are released into the liquid present in the processing region 220 (e.g., the polynucleotides are typically released into an amount of release liquid having a volume about the same as the void volume of the processing region 220). Typically, the polynucleotides are released into about 10 microliters or less (e.g., about 5 microliters or less, about 2.5 microliters or less) of liquid.

In certain embodiments, the ratio of the volume of original sample moved through the processing region 220 to the volume of liquid into which the polynucleotides are released is at least about 10 (e.g., at least about 50, at least about 100, at least about 250, at least about 500, at least about 1000). In some embodiments, polynucleotides from a sample having a volume of about 2 ml can be retained within the processing region, and released into about 4 microliters or

US 10,443,088 B1

15

less (e.g., about 3 microliters or less, about 2 microliters or less, about 1 microliter or less) of liquid.

The liquid into which the polynucleotides are released typically includes at least about 50% (e.g., at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **220**. The concentration of polynucleotides present in the release liquid may be higher than in the original sample because the volume of release liquid is typically less than the volume of the original liquid sample moved through the processing region. For example the concentration of polynucleotides in the release liquid may be at least about 10 times greater (e.g., at least about 25 times greater, at least about 100 times greater) than the concentration of polynucleotides in the sample introduced to device **200**. The concentration of inhibitors present in the liquid into which the polynucleotides are released is generally less than concentration of inhibitors in the original fluidic sample by an amount sufficient to increase the amplification efficiency for the polynucleotides.

The time interval between introducing the polynucleotide containing sample to processing region **220** and releasing the polynucleotides into the release liquid is typically about 15 minutes or less (e.g., about 10 minutes or less, about 5 minutes or less).

Liquid including the released polynucleotides may be removed from the processing region **220** as follows. Valves **210** and **234** are closed. Gates **238** and **258** are opened. Actuator **254** is actuated to generate pressure that moves liquid and polynucleotides from processing region **220**, into channel **230**, and toward outlet **236**. The liquid with polynucleotides can be removed using, for example, a syringe or automated sampling device. Depending upon the liquid in contact with retention member **216** during polynucleotide release, the solution with released polynucleotide may be neutralized with an amount of buffer (e.g., an equal volume of 25-50 mM Tris-HCl buffer pH 8.0).

While releasing the polynucleotides has been described as including a heating step, the polynucleotides may be released without heating. For example, in some embodiments, the liquid of reservoir **281** has an ionic strength, pH, surfactant concentration, composition, or combination thereof that releases the polynucleotides from the retention member.

While the polynucleotides have been described as being released into a single volume of liquid present within processing region **220**, other configurations can be used. For example, polynucleotides may be released with the concomitant (stepwise or continuous) introduction of fluid into and/or through processing region **220**. In such embodiments, the polynucleotides may be released into liquid having a volume of about 10 times or less (e.g., about 7.5 times or less, about 5 times or less, about 2.5 times or less, about 2 times or less) than the void volume of the processing region **220**.

While reservoirs **279**, **281** have been described as holding liquids between first and second gates, other configurations can be used. For example, liquid for each reservoir may be held within a pouch (e.g., a blister pack) isolated from network **201** by a generally impermeable membrane. The pouch is configured so that a user can rupture the membrane driving liquid into reservoirs **279**, **281** where actuators **244**, **248** can move the liquid during use.

While processing regions have been described as having microliter scale dimensions, other dimensions can be used. For example, processing regions with surfaces (e.g., particles) configured to preferentially retain polynucleotides as

16

opposed to inhibitors may have large volumes (e.g., many tens of microliters or more, at least about 1 milliliter or more). In some embodiments, the processing region has a bench-top scale.

While processing region **220** has been described as having a retention member formed of multiple surface-modified particles, other configurations can be used. For example, in some embodiments, processing region **220** includes a retention member configured as a porous member (e.g., a filter, a porous membrane, or a gel matrix) having multiple openings (e.g., pores and/or channels) through which polynucleotides pass. Surfaces of the porous member are modified to preferentially retain polynucleotides. Filter membranes available from, for example, Osmonics, are formed of polymers that may be surface-modified and used to retain polynucleotides within processing region **220**. In some embodiments, processing region **220** includes a retention member configured as a plurality of surfaces (e.g., walls or baffles) through which a sample passes. The walls or baffles are modified to preferentially retain polynucleotides.

While processing region **220** has been described as a component of a microfluidic network, other configurations can be used. For example, in some embodiments, the retention member can be removed from a processing region for processing elsewhere. For example, the retention member may be contacted with a mixture comprising polynucleotides and inhibitors in one location and then moved to another location at which the polynucleotides are removed from the retention member.

While reservoirs **275** have been shown as dispersed within a carrier, other configurations may be used. For example, reservoirs **275** can be encased within a flexible enclosure (e.g., a membrane, for example, an enclosure such as a sack). In some embodiments, reservoirs are loose within chamber **272**. In such embodiments, actuator **244** may include a porous member having pores too small to permit passage of reservoirs **275** but large enough to permit gas to exit chamber **272**.

Microfluidic devices with various components are described in U.S. provisional application No. 60/553,553 filed Mar. 17, 2004 by Parunak et al., which application is incorporated herein by reference.

While microfluidic device **300** has been described as configured to receive polynucleotides already released from cells, microfluidic devices can be configured to release polynucleotides from cells (e.g., by lysing the cells). For example, referring to FIGS. **4**, **5**, **6A**, and **6B**, a microfluidic device **300** includes a sample lysing chamber **302** in which cells are lysed to release polynucleotides therein. Microfluidic device **300** further includes substrate layers **L1-L3**, a microfluidic network **304** (only portions of which are seen in FIG. **4**), and liquid reagent reservoirs **R1-R4**. Liquid reagent reservoirs **R1-R4** hold liquid reagents (e.g., for processing sample material) and are connected to network **304** by reagent ports **RP1-RP4**.

Network **304** is substantially defined between layers **L2** and **L3** but extends in part between all three layers **L1-L3**. Microfluidic network **304** includes multiple components including channels **Ci**, valves **Vi**, double valves **V'i**, gates **Gi**, mixing gates **MGi**, vents **Hi**, gas actuators (e.g., pumps) **Pi**, a first processing region **B1**, a second processing region **B2**, detection zones **Di**, air vents **AVi**, and waste zones **Wi**.

Components of network **304** are typically thermally actuated. As seen in FIG. **7**, a heat source network **312** includes heat sources (e.g., resistive heat sources) having locations that correspond to components of microfluidic network **304**. For example, the locations of heat sources **HPi** correspond

US 10,443,088 B1

17

to the locations of actuators P_i , the locations of heat sources HG_i correspond to locations of gates G_i and mixing gates MG_i , the locations of heat sources HV_i correspond to the locations of valves V_i and double valves V'_i , and the locations of heat sources HD_i correspond to the locations of processing chambers D_i of network **304**. In use, the components of device **300** are disposed in thermal contact with corresponding heat sources of network **312**, which is typically operated using a processor as described above for device **200**. Heat source network **312** can be integral with or separate from device **300** as described for device **200**.

We next discuss components of microfluidic device **300**.

Air vents AV_i are components that allow gas (e.g., air) displaced by the movement of liquids within network **304** to be vented so that pressure buildup does not inhibit desired movement of the liquids. For example, air vent AV_2 permits liquid to move along channel C_{14} and into channel C_{16} by venting gas downstream of the liquid through vent AV_2 .

Valves V_i are components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). The valves V_i can have the same structure as valves of microfluidic device **200**.

As seen in FIGS. **8** and **9**, double valves V'_i are also components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Taking double valve V'_{11} of FIGS. **8** and **9** as an example, double valves V'_i include first and second masses **314**, **316** of a TRS (e.g., a eutectic alloy or wax) spaced apart from one another on either side of a channel (e.g., channel C_{14}). Typically, the TRS masses **314**, **316** are offset from one another (e.g., by a distance of about 50% of a width of the TRS masses or less). Material moving through the open valve passes between the first and second TRS masses **314**, **316**. Each TRS mass **314**, **316** is associated with a respective chamber **318**, **320**, which typically includes a gas (e.g., air).

The TRS masses **314**, **316** and chambers **318**, **320** of double valve V'_i are in thermal contact with a corresponding heat source HV'_{11} of heat source network **312**. Actuating heat source HV'_{11} causes TRS masses **314**, **316** to transition to a more mobile second state (e.g., a partially melted state) and increases the pressure of gas within chambers **318**, **320**. The gas pressure drives TRS masses **314**, **316** across channel C_{11} and closes valve HV'_{11} (FIG. **9**). Typically, masses **314**, **316** at least partially combine to form a mass **322** that obstructs channel C_{11} .

Returning to FIGS. **6A**, **6B**, gates G_i are components that have a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Gates G_i can have the same structure as described for gates of device **200**.

As seen in FIG. **10A-10D**, mixing gates MG_i are components that allow two volumes of liquid to be combined (e.g., mixed) within network **304**. Mixing gates MG_i are discussed further below.

Actuators P_i are components that provide a gas pressure to move material (e.g., sample material and/or reagent material) between one location of network **304** and another location. Actuators P_i can be the same as actuators of device **200**. For example, each actuator P_i includes a chamber with a mass **273** of TEM that can be heated to pressurize gas within the chamber. Each actuator P_i includes a corresponding gate G_i (e.g., gate G_2 of actuator P_1) that prevents liquid

18

from entering the chamber of the actuator. The gate is typically actuated (e.g., opened) to allow pressure created in the chamber of the actuator to enter the microfluidic network.

Waste chambers W_i are components that can receive waste (e.g., overflow) liquid resulting from the manipulation (e.g., movement and/or mixing) of liquids within network **304**. Typically, each waste chamber W_i has an associated air vent that allows gas displaced by liquid entering the chamber to be vented.

First processing region **B1** is a component that allows polynucleotides to be concentrated and/or separated from inhibitors of a sample. Processing region **B1** can be configured and operated as processing region **220** of device **200**. In some embodiments, first processing region **B1** includes a retention member (e.g., multiple particles (e.g., microspheres or beads), a porous member, multiple walls) having at least one surface modified with one or more ligands as described for processing region **220**. For example, the ligand can include one or more polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine), or polyethyleneimine. In some embodiments, particles of the retention member are disposed in lysing chamber **302** and are moved into processing region **B1** along with sample material.

Second processing region **B2** is a component that allows material (e.g., sample material) to be combined with compounds (e.g., reagents) for determining the presence of one or more polynucleotides. In some embodiments, the compounds include one or more PCR reagents (e.g., primers, control plasmids, and polymerase enzymes). Typically, the compounds are stored within processing region as one or more lyophilized particles (e.g., pellets). The particles generally have a room temperature (e.g., about 20° C.) shelf-life of at least about 6 months (e.g., at least about 12 months). Liquid entering the second processing region **B2** dissolves (e.g., reconstitutes) the lyophilized compounds.

Typically, the lyophilized particle(s) of processing region **B2** have an average volume of about 5 microliters or less (e.g., about 4 microliters or less, about 3 microliters or less, about 2 microliters or less). In some embodiments, the lyophilized particle(s) of processing region **B2** have an average diameter of about 4 mm or less (e.g., about 3 mm or less, about 2 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 2 microliters and an average diameter of about 1.35 mm.

Lyophilized particles for determining the presence of one or more polynucleotides typically include multiple compounds. In some embodiments, the lyophilized particles include one or more compounds used in a reaction for determining the presence of a polynucleotide and/or for increasing the concentration of the polynucleotide. For example, lyophilized particles can include one or more enzymes for amplifying the polynucleotide as by PCR.

We next discuss exemplary lyophilized particles that include exemplary reagents for the amplification of polynucleotides associated with group B *streptococcus* (GBS) bacteria. In some embodiments, the lyophilized particles include one or more of a cryoprotectant, one or more salts, one or more primers (e.g., GBS Primer F and/or GBS Primer R), one or more probes (e.g., GBS Probe—FAM), one or more internal control plasmids, one or more specificity controls (e.g., *Streptococcus pneumoniae* DNA as a control for PCR of GBS), one or more PCR reagents (e.g., dNTPs and/or dUTPs), one or more blocking or bulking agents (e.g., non-specific proteins (e.g., bovine serum albumin (BSA), RNaseA, or gelatin), and a polymerase (e.g., gly-

US 10,443,088 B1

19

erol-free Taq Polymerase). Of course, other components (e.g., other primers and/or specificity controls) can be used for amplification of other polynucleotides.

Cryoprotectants generally help increase the stability of the lyophilized particles and help prevent damage to other compounds of the particles (e.g., by preventing denaturation of enzymes during preparation and/or storage of the particles). In some embodiments, the cryoprotectant includes one or more sugars (e.g., one or more disaccharides (e.g., trehalose, melezitose, raffinose)) and/or one or more polyalcohols (e.g., mannitol, sorbitol).

Lyophilized particles can be prepared as desired. Typically, compounds of the lyophilized particles are combined with a solvent (e.g., water) to make a solution, which is then placed (e.g., in discrete aliquots (e.g., drops) such as by pipette) onto a chilled hydrophobic surface (e.g., a diamond film or a polytetrafluorethylene surface). In general, the temperature of the surface is reduced to near the temperature of liquid nitrogen (e.g., about -150°F . or less, about -200°F . or less, about -275°F . or less), such as by use of a cooling bath of a cryogenic agent directly underneath. It is to be noted that the solution is dispensed without contacting the cryogenic agent. The solution freezes as discrete particles. The frozen particles are subjected to a vacuum while still frozen for a pressure and time sufficient to remove the solvent (e.g., by sublimation) from the pellets.

In general, the concentrations of the compounds in the solution from which the particles are made is higher than when reconstituted in the microfluidic device. Typically, the ratio of the solution concentration to the reconstituted concentration is at least about 3 (e.g., at least about 4.5). In some embodiments, the ratio is about 6.

An exemplary solution for preparing lyophilized pellets for use in the amplification of polynucleotides indicative of the presence of GBS can be made by combining a cryoprotectant (e.g., 120 mg of trehalose as dry powder), a buffer solution (e.g., 48 microliters of a solution of 1M Tris at pH 8.4, 2.5M KCl, and 200 mM MgCl_2), a first primer (e.g., 1.92 microliters of 500 micromolar GBS Primer F (Invitrogen)), a second primer (e.g., 1.92 microliters of 500 micromolar GBS Primer R (Invitrogen)), a probe (e.g., 1.92 microliters of 250 micromolar GBS Probe—FAM (IDT/Biosearch Technologies)), an control probe (e.g., 1.92 microliters of 250 micromolar Cal Orange 560 (Biosearch Technologies)), a template plasmid (e.g., 0.6 microliters of a solution of 105 copies plasmid per microliter), a specificity control (e.g., 1.2 microliters of a solution of 10 nanograms per microliter (e.g., about 5,000,000 copies per microliter) *Streptococcus pneumoniae* DNA (ATCC)), PCR reagents (e.g., 4.8 microliters of a 100 millimolar solution of dNTPs (Epicenter) and 4 microliters of a 20 millimolar solution of dUTPs (Epicenter)), a bulking agent (e.g., 24 microliters of a 50 milligram per milliliter solution of BSA (Invitrogen)), a polymerase (e.g., 60 microliters of a 5 U per microliter solution of glycerol-free Taq Polymerase (Invitrogen/Ependorf)) and a solvent (e.g., water) to make about 400 microliters of solution. About 200 aliquots of about 2 microliters each of this solution are frozen and desolvated as described above to make 200 pellets. When reconstituted, the 200 particles make a PCR reagent solution having a total volume of about 2.4 milliliters.

As seen in FIG. 5, reagent reservoirs R_i are configured to hold liquid reagents (e.g., water, buffer solution, hydroxide solution) separated from network 304 until ready for use. Reservoirs R_1 include an enclosure 329 that defines a sealed space 330 for holding liquids. Each space 330 is separated from reagent port R_{Pi} and network 304 by a lower wall 333

20

of enclosure 329. A capping material 341 (e.g., a laminate, adhesive, or polymer layer) may overlie an upper wall of the enclosure.

A portion of enclosure 329 is formed as an actuation mechanism (e.g., a piercing member 331) oriented toward the lower wall 333 of each enclosure. When device 300 is to be used, reagent reservoirs R_i are actuated by depressing piercing member 331 to puncture wall 333. Piercing member 331 can be depressed by a user (e.g., with a thumb) or by the operating system used to operate device 300.

Wall 333 is typically formed of a material having a low vapor transmission rate (e.g., Aclar, a metallized (e.g. aluminum) laminate, a plastic, or a foil laminate) that can be ruptured or pierced. Reservoir 330 holds an amount of liquid suited for device 300. For example, the reservoir may hold up to about 200 microliters. The piercing member 331 may account for a portion (e.g., up to about 25%) of that volume.

In general, reservoirs R_i can be formed and filled as desired. For example, the upper wall of the enclosure can be sealed to the lower wall 333 (e.g., by adhesive and/or thermal sealing). Liquid can be introduced into the reservoir by, for example, an opening at the lower end of the piercing member 331. After filling, the opening can be sealed (e.g., by heat sealing through the localized application of heat or by the application of a sealing material (e.g., capping material 341)).

When wall 333 is punctured, fluid from the reservoir enters network 333. For example, as seen in FIGS. 5 and 6, liquid from reservoir R_2 enters network 304 by port RP_2 and travels along a channel C_2 . Gate G_3 prevents the liquid from passing along channel C_8 . Excess liquid passes along channel C_7 and into waste chamber W_2 . When the trailing edge of liquid from reservoir R_2 passes hydrophobic vent H_2 , pressure created within the reservoir is vented stopping further motion of the liquid. Consequently, network 304 receives an aliquot of liquid reagent having a volume defined by the volume of channel C_2 between a junction J_1 and a junction J_2 . When actuator P_1 is actuated, this aliquot of reagent is moved further within network 304. Reagent reservoirs R_1 , R_3 , and R_4 are associated with corresponding channels, hydrophobic vents, and actuators.

In the configuration shown, reagent reservoir R_1 typically holds a release liquid (e.g., a hydroxide solution as discussed above for device 200) for releasing polynucleotides retained within processing region B_1 . Reagent reservoir R_2 typically holds a wash liquid (e.g., a buffer solution as discussed above for device 200) for removing un-retained compounds (e.g., inhibitors) from processing region B_1 prior to releasing the polynucleotides. Reagent reservoir R_3 typically holds a neutralization buffer (e.g., 25-50 mM Tris-HCl buffer at pH 8.0). Reagent reservoir R_4 typically holds deionized water.

Lysing chamber 302 is divided into a primary lysing chamber 306 and a waste chamber 308. Material cannot pass from one of chambers 306, 308 into the other chamber without passing through at least a portion of network 304. Primary lysing chamber 306 includes a sample input port SP_1 for introducing sample to chamber 306, a sample output port SP_2 connecting chamber 306 to network 304, and lyophilized reagent LP that interact with sample material within chamber 306 as discussed below. Input port SP_1 includes a one way valve that permits material (e.g., sample material and gas) to enter chamber 306 but limits (e.g., prevents) material from exiting chamber 308 by port SP_1 . Typically, port SP_1 includes a fitting (e.g., a Luer fitting) configured to mate with a sample input device (e.g., a syringe) to form a gas-tight seal. Primary chamber 306

US 10,443,088 B1

21

typically has a volume of about 5 milliliters or less (e.g., about 4 milliliters or less). Prior to use, primary chamber 306 is typically filled with a gas (e.g., air).

Waste chamber 308 includes a waste portion W6 by which liquid can enter chamber 308 from network 304 and a vent 310 by which gas displaced by liquid entering chamber 308 can exit.

Lyophilized reagent particles LP of lysing chamber 302 include one or more compounds (e.g., reagents) configured to release polynucleotides from cells (e.g., by lysing the cells). For example, particles LP can include one or more enzymes configured to reduce (e.g., denature) proteins (e.g., proteinases, proteases (e.g., pronase), trypsin, proteinase K, phage lytic enzymes (e.g., PlyGBS)), lysozymes (e.g., a modified lysozyme such as ReadyLyse), cell specific enzymes (e.g., mutanolysin for lysing group B streptococci)).

In some embodiments, particles LP alternatively or additionally include components for retaining polynucleotides as compared to inhibitors. For example, particles LP can include multiple particles 218 surface modified with ligands as discussed above for device 200. Particles LP can include enzymes that reduce polynucleotides that might compete with a polynucleotide to be determined for binding sites on the surface modified particles. For example, to reduce RNA that might compete with DNA to be determined, particles LP may include an enzyme such as an RNAase (e.g., RNAaseA ISC BioExpress (Amresco)).

In an exemplary embodiment, particles LP cells include a cryoprotectant, particles modified with ligands configured to retain polynucleotides as compared to inhibitors, and one or more enzymes.

Typically, particles LP have an average volume of about 35 microliters or less (e.g., about 27.5 microliters or less, about 25 microliters or less, about 20 microliters or less). In some embodiments, the particles LP have an average diameter of about 8 mm or less (e.g., about 5 mm or less, about 4 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 20 microliters and an average diameter of about 3.5 mm.

Particles LP can be prepared as desired. Typically, the particles are prepared using a cryoprotectant and chilled hydrophobic surface as described above. For example, a solution for preparing particles LP can be prepared by combining a cryoprotectant (e.g., 6 grams of trehalose), a plurality of particles modified with ligands (e.g., about 2 milliliters of a suspension of carboxylate modified particles with poly-D-lysine ligands), a protease (e.g., 400 milligrams of pronase), an RNAase (e.g., 30 milligrams of RNAaseA (activity of 120 U per milligram), an enzyme that digests peptidoglycan (e.g., ReadyLyse (e.g., 160 microliters of a 30000 U per microliter solution of ReadyLyse)), a cell specific enzyme (e.g., mutanolysin (e.g., 200 microliters of a 50 U per microliter solution of mutanolysin), and a solvent (e.g., water) to make about 20 milliliters. About 1000 aliquots of about 20 microliters each of this solution are frozen and desolvated as described above to make 1000 pellets. When reconstituted, the pellets are typically used to make a total of about 200 milliliters of solution.

In use, device 300 can be operated as follows. Valves Vi and Vi' of network 304 are configured in the open state. Gates Gi and mixing gates MG_i of network 304 are configured in the closed state. Reagent ports R1-R4 are depressed to introduce liquid reagents into network 304 as discussed above. A sample is introduced to lysing chamber 302 via port SP1 and combined with lyophilized particles LP within primary lysing chamber 306. Typically, the sample includes

22

a combination of particles (e.g., cells) and a buffer solution. For example, an exemplary sample includes about 2 parts whole blood to 3 about parts buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% SDS). Another exemplary sample includes group B streptococci and a buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% Triton X-100).

In general, the volume of sample introduced is smaller than the total volume of primary lysing chamber 306. For example, the volume of sample may be about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. A typical sample has a volume of about 3 milliliters or less (e.g., about 1.5 milliliters or less). A volume of gas (e.g., air) is generally introduced to primary chamber 306 along with the sample. Typically, the volume of gas introduced is about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. The volume of sample and gas combine to pressurize the gas already present within chamber 306. Valve 307 of port SP1 prevents gas from exiting chamber 306. Because gates G3, G4, G8, and G10 are in the closed state, the pressurized sample is prevented from entering network 304 via port SP2.

The sample dissolves particles LP in chamber 306. Reconstituted lysing reagents (e.g., ReadyLyse, mutanolysin) begin to lyse cells of the sample releasing polynucleotides. Other reagents (e.g., protease enzymes such as pronase) begin to reduce or denature inhibitors (e.g., proteins) within the sample. Polynucleotides from the sample begin to associate with (e.g., bind to) ligands of particles 218 released from particles LP. Typically, the sample within chamber 306 is heated (e.g., to at least about 50° C., to at least about 60° C.) for a period of time (e.g., for about 15 minutes or less, about 10 minutes or less, about 7 minutes or less) while lysing occurs. In some embodiments, optical energy is used at least in part to heat contents of lysing chamber 306. For example, the operating system used to operate device 300 can include a light source (e.g., a lamp primarily emitting light in the infrared) disposed in thermal and optical contact with chamber 306. Chamber 306 includes a temperature sensor TS used to monitor the temperature of the sample within chamber 306. The lamp output is increased or decreased based on the temperature determined with sensor TS.

Continuing with the operation of device 300, G2 is actuated (e.g., opened) providing a path between port SP2 of primary lysing chamber 306 and port W6 of lysing waste chamber 308. The path extends along channel C9, channel C8, through processing region B1, and channel C11. Pressure within chamber 306 drives the lysed sample material (containing lysate, polynucleotides bound to particles 218, and other sample components) along the pathway. Particles 218 (with polynucleotides) are retained within processing region B1 (e.g., by a filter) while the liquid and other components of the sample flow into waste chamber 308. After a period of time (e.g., between about 2 and about 5 minutes), the pressure in lysing chamber 306 is vented by opening gate G1 to create a second pathway between ports SP2 and W6. Double valves V1' and V8' are closed to isolate lysing chamber 302 from network 304.

Operation of device 300 continues by actuating pump P1 and opening gates G2, G3 and G9. Pump P1 drives wash liquid in channel C2 downstream of junction J1 through processing region B1 and into waste chamber W5. The wash liquid removes inhibitors and other compounds not retained by particles 218 from processing region B1. When the trailing edge of the wash liquid (e.g., the upstream interface) passes hydrophobic vent H14, the pressure from actuator P1

US 10,443,088 B1

23

vents from network **304**, stopping further motion of the liquid. Double valves **V2'** and **V9'** are closed.

Operation continues by actuating pump **P2** and opening gates **G6**, **G4** and **G8** to move release liquid from reagent reservoir **R1** into processing region **B1** and into contact with particles **218**. Air vent **AV1** vents pressure ahead of the moving release liquid. Hydrophobic vent **H6** vents pressure behind the trailing edge of the release liquid stopping further motion of the release liquid. Double valves **V6'** and **V10'** are closed.

Operation continues by heating processing region **B1** (e.g., by heating particles **218**) to release the polynucleotides from particles **218**. The particles can be heated as described above for device **200**. Typically, the release liquid includes about 15 mM hydroxide (e.g., NaOH solution) and the particles are heated to about 70° C. for about 2 minutes to release the polynucleotides from the particles **218**.

Operation continues by actuating pump **P3** and opening gates **G5** and **G10** to move release liquid from process region **B1** downstream. Air vent **AV2** vents gas pressure downstream of the release liquid allowing the liquid to move into channel **C16**. Hydrophobic vent **H8** vents pressure from upstream of the release liquid stopping further movement. Double valve **V11'** and valve **V14** are closed.

Referring to FIG. **10A-10D**, mixing gate **MG11** is used to mix a portion of release liquid including polynucleotides released from particles **218** and neutralization buffer from reagent reservoir **R3**. FIG. **10A** shows the mixing gate **MG11** region prior to depressing reagent reservoir **R3** to introduce the neutralization buffer into network **304**. FIG. **10B** shows the mixing gate **MG11** region, after the neutralization buffer has been introduced into channels **C13** and **C12**. Double valve **V13'** is closed to isolate network **304** from reagent reservoir **R3**. Double valve **V12'** is closed to isolate network **304** from waste chamber **W3**. The neutralization buffer contacts one side of a mass **324** of TRS of gate **MG11**.

FIG. **10c** shows the mixing gate **MG11** region after release liquid has been moved into channel **C16**. The dimensions of microfluidic network **304** (e.g., the channel dimensions and the position of hydrophobic vent **H8**) are configured so that the portion of release liquid positioned between junctions **J3** and **J4** of channels **C16** and **C14** corresponds approximately to the volume of liquid in contact with particles **218** during the release step. In some embodiments, the volume of liquid positioned between junctions **J3** and **J4** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J3** and **J4** is about 1.75 microliters. Typically, the liquid between junctions **J3** and **J4** includes at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **B1**. Valve **V14** is closed to isolate network **304** from air vent **AV2**.

Before actuating mixing gate **MG11**, the release liquid at junction **J4** and the neutralization buffer at a junction **J6** between channels **C13** and **C12** are separated only by mass **324** of TRS (e.g., the liquids are not spaced apart by a volume of gas). To combine the release liquid and neutralization buffer, pump **P4** and gates **G12**, **G13**, and **MG11** are actuated. Pump **P4** drives the volume of neutralization liquid between junctions **J5** and **J6** and the volume of release liquid between junctions **J4** and **J3** into mixing channel **C15** (FIG. **10D**). Mass **324** of TRS typically disperses and/or melts allowing the two liquids to combine. The combined liquids include a downstream interface **335** (formed by junction **J3**)

24

and an upstream interface (formed by junction **J5**). The presence of these interfaces allows more efficient mixing (e.g., recirculation of the combined liquid) than if the interfaces were not present. As seen in FIG. **10D**, mixing typically begins near the interface between the two liquids. Mixing channel **C15** is typically at least about as long (e.g., at least about twice as long) as a total length of the combined liquids within the channel.

The volume of neutralization buffer combined with the release liquid is determined by the channel dimensions between junction **J5** and **J6**. Typically, the volume of combined neutralization liquid is about the same as the volume of combined release liquid. In some embodiments, the volume of liquid positioned between junctions **J5** and **J6** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J5** and **J6** is about 2.25 microliters (e.g., the total volume of release liquid and neutralization buffer is about 4 microliters).

Returning to FIGS. **6A**, **6B**, the combined release liquid and neutralization buffer move along mixing channel **C15** and into channel **C32** (vented downstream by air vent **AV8**). Motion continues until the upstream interface of the combined liquids passes hydrophobic vent **H11**, which vents pressure from actuator **P4** stopping further motion of the combined liquids.

Continuing with operation of device **300**, actuator **P5** and gates **G14**, **G15** and **G17** are actuated to dissolve the lyophilized PCR particle present in second processing region **B2** in water from reagent reservoir **R4**. Hydrophobic vent **H10** vents pressure from actuator **P5** upstream of the water stopping further motion. Dissolution of a PCR-reagent pellet typically occurs in about 2 minutes or less (e.g., in about 1 minute or less). Valve **V17** is closed.

Continuing with operation of device **300**, actuator **P6** and gate **G16** are actuated to drive the dissolved compounds of the lyophilized particle from processing region **B2** into channel **C31**, where the dissolved reagents mix to form a homogenous dissolved lyophilized particle solution. Actuator **P6** moves the solution into channels **C35** and **C33** (vented downstream by air vent **AV5**). Hydrophobic vent **H9** vents pressure generated by actuator **P6** upstream of the solution stopping further motion. Valves **V18**, **V19**, **V20'**, and **V22'** are closed.

Continuing with operation of device **300**, actuator **P7** and gates **G18**, **MG20** and **G22** are actuated to combine (e.g., mix) a portion of neutralized release liquid in channel **32** between gate **MG20** and gate **G22** and a portion of the dissolved lyophilized particle solution in channel **C35** between gate **G18** and **MG20**. The combined liquids travel along a mixing channel **C37** and into detection region **D2**. An air vent **AV3** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H13**, the pressure from actuator **P7** is vented and the combined liquids are positioned within detection region **D2**.

Actuator **P8** and gates **MG2**, **G23**, and **G19** are actuated to combine a portion of water from reagent reservoir **R4** between **MG2** and gate **G23** with a second portion of the dissolved lyophilized particle solution in channel **C33** between gate **G19** and **MG2**. The combined liquids travel along a mixing channel **C41** and into detection region **D1**. An air vent **AV4** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H12**, the pressure from actuator **P8** is vented and the combined liquids are positioned within detection region **D1**.

US 10,443,088 B1

25

Continuing with operation of device **300**, double valves **V26'** and **V27'** are closed to isolate detection region **D1** from network **304** and double valves **V24'** and **V25'** are closed to isolate detection region **D2** from network **304**. The contents of each detection region (neutralized release liquid with sample polynucleotides in detection region **D2** with PCR reagents from dissolved lyophilized particle solution and deionized water with PCR reagents from dissolved lyophilized particle solution in detection region **D1**) are subjected to heating and cooling steps to amplify polynucleotides (if present in detection region **D2**). The double valves of each detection region prevent evaporation of the detection region contents during heating. The amplified polynucleotides are typically detected using fluorescence detection.

While reservoirs have been shown as having a piercing member formed of a wall of the reservoir, other configurations are possible. For example, in some embodiments, the reservoir includes a needle-like piercing member that extends through an upper wall of the reservoir into the sealed space toward a lower wall of the reservoir. The upper wall of the reservoir may be sealed at the needle-like piercing member (e.g., with an adhesive, an epoxy). In use, the upper wall is depressed driving the piercing member through the lower wall forcing liquid in the sealed space to enter a microfluidic network.

While reservoirs have been described as including an actuation mechanism (e.g., a piercing member), other configurations are possible. For example, in some embodiments, a lower wall of the sealed space of the reservoir includes a weakened portion that overlies an opening to a microfluidic network. The lower wall material (e.g., laminate, polymer film, or foil) that overlies the opening is thick enough to prevent loss of the liquid within the sealed space but thin enough to rupture upon the application of pressure to the liquid therein. Typically, the material overlying the opening is thinner than the adjacent material. Alternatively, or in addition, the weakened material can be formed by leaving this material relatively unsupported as compared to the surrounding material of the lower wall.

While reservoirs have been described as having a sealed space formed in part by a wall of the sealed space, other configurations are possible. For example, referring to FIG. **11A**, a reservoir includes a plunger-like actuation mechanism (e.g., a piercing member **342**) and a gasket-like sealed space **343** having upper and lower layers **344**, **345** respectively (e.g., upper and lower laminate layers). Liquid is sealed between the upper and lower layers. The sealed space can be surrounded by a supporting structure **346** (e.g., a toroidal gasket) that supports the sealed space at its upper and lower peripheral surfaces.

Referring to FIG. **11B**, piercing member **342** is shown as being depressed until the piercing member **342** has pierced both the upper and lower layers bringing the liquid into communication with the microfluidic network. A vent **346** adjacent the plunger allows gas trapped between the piercing member and the upper layer of the sealed space to escape without being forced into the microfluidic network.

Referring to FIG. **11C**, piercing member **342** is shown as fully actuated. A portion of the piercing member has displaced a corresponding volume of liquid from the sealed space and introduced the predetermined volume of liquid into the microfluidic device.

While the reservoirs have been described as having a sealed space that may be stationary with respect to a piercing member, other configurations are possible. For example, FIG. **12A** illustrates a reservoir having a sealed space **347**

26

that is secured with (e.g., integral with) respect to an actuation mechanism having a movable member **348** (e.g., a plunger) and a piercing member **349** supported by a piercing member support **350** that are stationary with respect to the sealed space. Typically, the sealed space is defined by a cavity within the movable member and a lower wall **351** that seals liquid within the sealed space. Piercing member is configured to rupture the lower wall when the movable member is depressed. Piercing member support has a shape generally complementary to the cavity of the movable member. Piercing member support includes a channel **352** connected to a microfluidic network to allow fluid released from the enclosed space to enter the microfluidic network.

Referring to FIG. **12B**, the movable member has been depressed so that the piercing member has just ruptured the lower layer of the sealed space. Referring to FIG. **12C**, the reservoir has been fully depressed onto the piercing member and piercing member support. The volume of fluid displaced from the reservoir generally corresponds to the volume of the piercing member support that enters the enclosed space. A channel **353** allows air displaced by the moveable member to exit.

While reservoirs have been described as having a piercing member that is secured with respect to some portion of the reservoir, other configurations are possible. For example, referring to FIG. **13**, a reservoir includes an actuation mechanism **354** (e.g., a piercing member such as a needle-like piercing member) that is unsecured with respect to the reservoir. A sealed space **355** of the reservoir is defined by an upper wall **356** and includes a channel **357** extending through a portion of a substrate **361** in which a microfluidic network is defined. A lower wall **358** of the sealed space separates the sealed space from a channel **359** of the microfluidic network. The piercing member occupies the channel **357** of the sealed space so that the piercing tip **360** of the piercing member rests against the lower wall **358**. Depressing the upper wall **356** of the reservoir drives the piercing member **354** through the lower wall and forces liquid within the sealed space into the microfluidic network.

As another example, FIGS. **14A** and **14B** illustrate a reservoir including an actuation mechanism (e.g., a piercing member) that is initially secured to an interior of an upper wall of the reservoir but separates at least partially from the upper wall upon actuation of the reservoir.

As yet another example, FIGS. **15A** and **15B** illustrate a reservoir including a piercing member **364** that is initially secured to an interior **365** of an upper wall **366** of the reservoir but substantially separates (e.g., completely separates) from the upper wall upon actuation of the reservoir.

While reservoirs have been described as having an enclosed space that is fixed or otherwise integral with a portion of the reservoir, other configurations are possible. For example, referring to FIG. **16**, a reservoir includes a capsule-like enclosed space **367** defined by an outer wall **368**. The outer wall is generally formed of a material having a low vapor transmission rate. Reservoir also includes an actuation mechanism having a moveable member **369** with a piercing member **370** that pierces the enclosed space to release liquid therein. The liquid passes along a channel **372** leading to a microfluidic network. A channel **371** allows gas (e.g., air) otherwise trapped by the movable member to exit.

While reservoirs have been described as generally overlying an inlet to a microfluidic network, other configurations are possible. For example, referring to FIG. **17**, a reservoir includes an enclosed space **373** in which liquid is stored and a connecting portion **374** connected to an inlet **376** of a microfluidic network. The enclosed space **373** and connect-

US 10,443,088 B1

27

ing portion **374** are separated by a rupturable seal **375** (e.g., a weak seal). In general, the rupturable seal **375** prevents liquid or vapor from exiting the enclosed space. However, upon the application of pressure to the liquid (e.g., by depressing a wall **377** of the enclosed space), the rupturable seal **375** ruptures allowing the liquid to pass through the weak seal to the connecting portion and into the microfluidic network **378**.

A still further embodiment of a reservoir with a piercing member is shown in FIG. **27A**, which shows a reservoir **2701** having an outer shell **2703** and a piercing element **2704** that are both made of the same piece of material. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2704** is generally conical in shape, with the apex adjacent to a membrane **2702**; its apex preferably does not exceed 0.040". The piercing element will puncture membrane **2702** and release liquid from reservoir **2701** when the outer shell is depressed. Representative dimensions are shown on FIG. **27A**. The reservoir may be constructed so that the upper surface is level, with a flat protective piece **2705** covering the base of the conical shape of piercing element **2704**.

Yet another embodiment of a reservoir with a piercing member is shown in FIG. **27B**, showing a reservoir **2711** having a single-piece outer shell **2712** and piercing element **2714**. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2714** can be frustoconical in shape, with its narrower side adjacent to membrane **2713**. Alternatively, piercing element **2714** can comprise several separate piercing elements, arranged within a conical space. Preferably there are four such piercing elements where multiple elements are present.

It is to be understood that the dimensions of the reservoir, piercing element, shell and moulding shown in FIGS. **27A** and **27B** as decimal quantities in inches are exemplary. In particular, the dimensions are such that the shell does not collapse under its own weight and is not so as strong to prohibit depression of the piercing member when required during operation of the device.

Furthermore, the materials of the various embodiments are also chosen so that the device has a shelf-life of about a year. By this it is meant that the thickness of the various materials are such that they resist loss, through means such as diffusion, of 10% of the liquid volume contained therein over a desired shelf-life period.

Preferably the volume of the reservoir is around 150 μ L before a shell is depressed. Upon depression of a shell, the volume is preferably deformed to around half its original volume.

While devices for processing samples have been described as having a generally planar configuration, other configurations can be used. For example, referring to FIG. **18**, a device **700** configured to process a polynucleotide-containing sample, such as to prepare the sample for amplification of the polynucleotides, has a generally tube-like or vial-like configuration. Device **700** includes a sample reservoir **704**, a reagent reservoir **706**, a gas pressure generator **708**, a closure (e.g., a cap **710**), and a processing region **702** including a retention member **704** having a plurality of particles (e.g. carboxylate beads **705** surface-modified with a ligand, e.g., poly-L-lysine and/or poly-D-lysine, or poly-ethyleneimine). Retention member **705** and beads **705** may share any or all properties of retention member **216** and

28

surface-modified particles **218**. Device **700** also includes an opening **716** and a valve, e.g., a thermally actuated valve **714** for opening and closing opening **716**.

In use, a polynucleotide-containing sample is added to sample reservoir **704**. Typical sample amounts range from about 100 μ L to about 2 mL, although greater or smaller amounts may be used.

Reagent reservoir **706** may be provided to users of device **700** with pre-loaded reagent. Alternatively, device **700** may be configured so that users add reagent to device **700**. In any event, the reagents may include, e.g., NaOH solutions and/or buffer solutions such as any of such solutions discussed herein.

Once sample and, if necessary, reagent have been added to device **700**, cap **710** is closed to prevent evaporation of sample and reagent materials.

Referring also to FIG. **19**, an operator **718** is configured to operate device **700**. Operator **718** includes a first heat source **720** and a second heat source **722**. First heat source **720** heats sample present within sample reservoir **704**, such as to lyse cells of the polynucleotide-containing sample to prepare free polynucleotides.

Device **700** may also include an enzyme reservoir **712** comprising an enzyme, e.g., a protease such as pronase, configured to cleave peptide bonds of polypeptides present in the polynucleotide-containing sample. Enzyme reservoir **712** may be provided to users of device **700** with pre-loaded enzyme. Alternatively, device **700** may be configured so that users add enzyme to device **700**.

Device **700** may be used to reduce the amount of inhibitors present relative to the amount of polynucleotides to be determined. Thus, the sample is eluted through processing region **702** to contact constituents of the sample with beads **705**. Beads **705** retain polynucleotides of the sample as compared to inhibitors as described elsewhere herein. With valve **714** in the open state, sample constituents not retained in processing region **702** exit device **700** via the opening.

Once the polynucleotide-containing sample has eluted through processing region **702**, an amount of reagent, e.g., a wash solution, e.g., a buffer such as Tris-EDTA pH 8.0 with 1% Triton X 100 is eluted through processing region **702**. The wash solution is generally stored in reagent reservoir **706**, which may include a valve configured to release an amount of wash solution. The wash solution elutes remaining polynucleotide-containing sample and inhibitors without eluting retained polynucleotides.

Once inhibitors have been separated from retained polynucleotides, the polynucleotides are released from beads **705**. In some embodiments, polynucleotides are released by contacting the beads **705** with a release solution, e.g., a NaOH solution or buffer solution having a pH different from that of the wash solution. Alternatively, or in combination, beads **705** with retained polynucleotides are heated, such as by using second heat source **722** of operator **718**. When heat is used to release the polynucleotides, the release solution may be identical with the wash solution.

Gas pressure generator **708** may be used to expel an amount of release solution with released polynucleotides from device **700**. Gas pressure generator and/or operator **718** may include a heat source to heat gas present within generator **708**. The heated gas expands and provides the gas pressure to expel sample. In some embodiments, and whether or not thermally generated gas pressure is used, gas pressure generator **708** is configured to expel a predetermined volume of material. Typically, the amount of expelled

US 10,443,088 B1

29

solution is less than about 500 μL , less than about 250 μL , less than about 100 μL , less than about 50 μL , e.g., less than about 25 μL .

EXAMPLES

The following Examples are illustrative and are not intended to be limiting.

Example 1 Preparing Retention Member

Carboxylate surface magnetic beads (Sera-Mag Magnetic Carboxylate modified, Part #3008050250, Seradyn) at a concentration of about 1011 mL^{-1} were activated for 30 minutes using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in a pH 6.1 500 mM 2-(N-Morpholinio)-ethanesulfonic acid (MES) buffer solution. Activated beads were incubated with 3000 Da or 300,000 Da average molecular weight poly-L-lysine (PLL). After 2 washes to remove unbound PLL, beads were ready for use.

Example 2 Microfluidic Device

Referring to FIGS. 20 and 21, a microfluidic device 300 was fabricated to demonstrate separation of polynucleotides from inhibitors. Device 300 comprises first and second substrate portions 302', 304', which respectively comprise first and second layers 302a', 302b' and 304a', 304b'. First and second layers 302a', 302b' define a channel 306' comprising an inlet 310' and an outlet 312'. First and second layers 304a', 304b' define a channel 308' comprising an inlet 314' and an outlet 316'. First and second substrate portions 302', 304' were mated using adhesive 324' so that outlet 312' communicated with inlet 314' with a filter 318' positioned therebetween. A portion of outlet 312' was filed with the activated beads prepared above to provide a processing region 320' comprising a retention member (the beads). A pipette 322' (FIG. 22) secured by adhesive 326' facilitated sample introduction.

In use, sample introduced via inlet 310' passed along channel and through processing region 320'. Excess sample material passed along channel 308' and exited device 300' via outlet 316'. Polynucleotides were preferentially retained by the beads as compared to inhibitors. Once sample had been introduced, additional liquids, e.g., a wash liquid and/or a liquid for use in releasing the retained polynucleotides were introduced via inlet 326'.

Example 3 Retention of DNA

Retention of polynucleotides by the poly-L-lysine modified beads of device 300' was demonstrated by preparing respective devices comprising processing regions having a volume of about 1 μL including about 1000 beads. The beads were modified with poly-L-lysine of between about 15,000 and 30,000 Da. Each processing region was filled with a liquid comprising herring sperm DNA (about 20 μL of sample with a concentration of about 20 mg/mL) thereby placing the beads and liquid in contact. After the liquid and beads had been in contact for 10 minutes, the liquid was removed from each processing region and subjected to quantitative real-time PCR to determine the amount of herring sperm DNA present in the liquid.

Two controls were performed. First, an otherwise identical processing region was packed with unmodified beads, i.e., beads that were identical with the poly-L-lysine beads

30

except for the activation and poly-L-lysine incubation steps. The liquid comprising herring sperm DNA was contacted with these beads, allowed to stand for 10 minutes, removed, and subjected to quantitative real-time PCR. Second, the liquid comprising the herring sperm DNA ("the unprocessed liquid") was subjected to quantitative real-time PCR.

Referring to FIG. 22, the first and second controls exhibited essentially identical responses indicating the presence of herring sperm DNA in the liquid contacted with the unmodified beads and in the unprocessed liquid. The liquid that had contacted the 3,000 poly-L-lysine beads exhibited a lower response indicating that the modified beads had retained substantially all of the herring sperm DNA. The PCR response of the liquid that had contacted the 300,000 Da poly-L-lysine beads exhibited an amplification response that was at least about 50% greater than for the 3,000 Da beads indicating that the lower molecular weight surface modification was more efficient at retaining the herring sperm DNA.

Example 4 Releasing DNA from Poly-L-Lysine Modified Beads

Devices having processing regions were packed with 3,000 Da poly-L-lysine modified beads. Liquid comprising polynucleotides obtained from group B streptococci (GBS) was contacted with the beads and incubated for 10 minutes as above for the herring sperm DNA. This liquid had been obtained by subjecting about 10,000 GBS bacteria in 10 μL of 20 mM Tris pH 8, 1 mM EDTA, 1% Triton X-100 buffer to thermal lysing at 97° C. for 3 min.

After 10 minutes, the liquid in contact with the beads was removed by flowing about 10 μL of wash solution (Tris-EDTA pH 8.0 with 1% Triton X 100) through the processing region. Subsequently, about 1 μL of 5 mM NaOH solution was added to the processing region. This process left the packed processing region filled with the NaOH solution in contact with the beads. The solution in contact with the beads was heated to 95° C. After 5 minutes of heating at 95° C., the solution in contact with the beads was removed by eluting the processing region with a volume of solution equal to three times the void volume of the processing region.

Referring to FIG. 23, five aliquots of solution were subjected to quantitative real-time PCR amplification. Aliquots E1, E2, and E3 each contained about 1 μL of liquid. Aliquot L corresponds to liquid of the original sample that had passed through the processing region. Aliquot W was liquid obtained from wash solution without heating. Aliquot E1 corresponds to the dead volume of device 300, about equal to the volume of channel 308. Thus, liquid of aliquot E1 was present in channel 308 and not in contact with the beads during heating. This liquid had passed through the processing region prior to heating. Aliquot E2 comprises liquid that was present within the processing region and in contact with the beads during heating. Aliquot E3 comprises liquid used to remove aliquot E2 from the processing region.

As seen in FIG. 23, more than 65% of the GBS DNA present in the initial sample was retained by and released from the beads (Aliquot E2). Aliquot E2 also demonstrates the release of more than 80% of the DNA that had been retained by the beads. Less than about 18% of the GBS DNA passed through the processing region without being cap-

US 10,443,088 B1

31

tured. The wash solution without heating comprised less than 5% of the GBS DNA (Aliquot W).

Example 5 Separation of Polynucleotides and Inhibitors

Buccal cells from the lining of the cheeks provide a source of human genetic material (DNA) that may be used for single nucleotide polymorphism (SNP) detection. A sample comprising buccal cells was subjected to thermal lysing to release DNA from within the cells. Device 300 was used to separate the DNA from concomitant inhibitors as described above. A cleaned-up sample corresponding to aliquot E2 of FIG. 23 was subjected to polymerase chain reaction. A control or crude sample as obtained from the thermal lysing was also amplified.

Referring to FIG. 24, the cleaned-up sample exhibited substantially higher PCR response in fewer cycles than did the control sample. For example, the clean-up sample exceeded a response of 20 within 32 cycles whereas the control sample required about 45 cycles to achieve the sample response.

Blood acts as a sample matrix in variety of diagnostic tests including detection of infectious disease agents, cancer markers and other genetic markers. Hemoglobin present in blood samples is a documented potent inhibitor of PCR. Two 5 ml blood samples were lysed in 20 mM Tris pH 8, 1 mM EDTA, 1% SDS buffer and introduced to respective devices 300, which were operated as described above to prepare two clean-up samples. A third 5 ml blood sample was lysed and prepared using a commercial DNA extraction method Puregene, Gentra Systems, MN. The respective cleaned-up samples and sample subjected to the commercial extraction method were used for a Allelic discrimination analysis (CYP2D6*4 reagents, Applied Biosystems, CA). Each sample contained an amount of DNA corresponding to about 1 ml of blood.

Referring to FIG. 25, the cleaned-up and commercially extracted samples exhibited similar PCR response demonstrating that the processing region of device 300' efficiently removed inhibitors from the blood samples.

Example 6 Protease Resistant Retention Member

The preparation of polynucleotide samples for further processing often includes subjecting the samples to protease treatment in which a protease cleaves peptide bonds of proteins in the sample. An exemplary protease is pronase, a mixture of endo- and exo-proteases. Pronase cleaves most peptide bonds. Certain ligands, such as poly-L-lysine are susceptible to rupture by pronase and other proteases. Thus, if samples are generally not subjected to protease treatment in the presence of the retention member if the ligands bound thereto are susceptible to the proteases.

Poly-D-lysine, the dextro enantiomer of poly-lysine resists cleavage by pronase and other proteases. The ability of a retention member comprising bound poly-D-lysine to retain DNA even when subjected to a protease treatment was studied.

Eight (8) samples were prepared. A first group of 4 samples contained 1000 GBS cells in 10 µl buffer. A second group of 4 samples contained 100 GBS cells in 10 µl buffer. Each of the 8 samples was heated to 97° C. for 3 min to lyse the GBS cells. Four (4) sample sets were created from the heated samples. Each sample set contained 1 sample from each of the first and second groups. The samples of each sample sets were treated as follows.

32

Referring to FIG. 26A, the samples of sample set 1 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 2 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 3 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 4 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

As seen in FIG. 26B, an average of more than 80% of DNA from the GBS cells was recovered using sample set 4 in which the samples were contacted with poly-D-lysine modified beads and subjected to pronase incubation in the presence of the beads without protease inactivation. The

US 10,443,088 B1

33

recovery efficiency for sample set 4 is more than twice as high as for any of the other samples. Specifically, the recovery efficiencies for sample sets 1, 2, 3, and 4, were 29%, 32%, 14%, and 81.5%, respectively. The efficiencies demonstrate that high recovery efficiencies can be obtained for samples subjected to protease incubation in the presence of a retention member that retains DNA.

Other embodiments are within the claims.

What is claimed is:

1. A method for processing a polynucleotide-containing sample, the method comprising:

retaining polynucleotides from a sample on a plurality of binding particles in a process chamber under a first set of conditions, wherein the retaining step comprises binding the polynucleotides to the surfaces of the plurality of binding particles comprising a poly-cationic substance, wherein the sample has a volume from 0.5 microliters to 3 milliliters;

wherein the first set of conditions includes a first pH of about 8.5 or less and a first temperature, wherein the first temperature is between about 30° C. and about 50° C.;

releasing the polynucleotide from the plurality of binding particles under a second set of conditions; and

wherein the second set of conditions includes increasing the pH to a second pH by addition of a hydroxide solution and increasing the temperature to a second temperature, wherein the second temperature is between about 80° C. and about 100° C.

2. The method of claim 1, wherein the second pH is about 11.3 or greater.

3. The method of claim 1, wherein the poly-cationic substance is covalently bound to the surfaces of the binding particles.

4. The method of claim 1, wherein the binding particles comprise one or more carboxylic groups to provide an attachment point for the poly-cationic substance.

5. The method of claim 4, further comprising treating magnetic beads with NHS and MAC to form the binding particles.

6. The method of claim 1, wherein the heating step comprises heating the plurality of binding particles in the presence of a liquid, wherein the second temperature is insufficient to boil the liquid.

7. The method of claim 1, further comprising maintaining the second temperature for between about 1 and 10 minutes.

8. The method of claim 1, wherein the sample is heated for about 15 minutes or less while contacting the binding particles with the basic solution thereby releasing the polynucleotides.

9. The method of claim 1, wherein the poly-cationic substance has a molecular weight of less than about 30,000 Da.

10. The method of claim 1, wherein the poly-cationic substance has a molecular weight of less than about 800 Da.

34

11. The method of claim 1, wherein the ratio of the volume of the sample in the process chamber to the volume of liquid into which the polynucleotides are released is at least about 10.

12. The method of claim 1, wherein the particles occupy about 75 percent or less of the total volume of the process chamber.

13. A method for processing a polynucleotide-containing sample, the method comprising:

retaining polynucleotides from a sample on a plurality of binding particles in a process chamber under a first set of conditions, wherein the retaining step comprises binding the polynucleotides to the surfaces of the plurality of binding particles comprising a poly-cationic substance; wherein the sample has a volume from 0.5 microliters to 3 milliliters;

wherein the first set of conditions includes a first pH of about 8.5 or less and a first temperature, wherein the first temperature is about 60° C.;

releasing the polynucleotide from the plurality of binding particles under a second set of conditions; and

wherein the second set of conditions includes increasing the pH to a second pH by addition of a hydroxide solution and increasing the temperature to a second temperature, wherein the second temperature is between about 80° C. and about 100° C.

14. The method of claim 13, wherein the second pH is about 11.3 or greater.

15. The method of claim 13, wherein the poly-cationic substance is covalently bound to the surfaces of the binding particles.

16. The method of claim 13, wherein the binding particles comprise one or more carboxylic groups to provide an attachment point for the poly-cationic substance.

17. The method of claim 16, further comprising treating magnetic particles with NHS and EDAC to form the binding particles.

18. The method of claim 13, further comprising maintaining the second temperature for between about 1 and 10 minutes.

19. The method of claim 13, wherein the sample is heated for about 15 minutes or less while contacting the binding particles with the basic solution thereby releasing the polynucleotides.

20. The method of claim 13, wherein the poly-cationic substance has a molecular weight of less than about 30,000 Da.

21. The method of claim 13, wherein the poly-cationic substance has a molecular weight of less than about 800 Da.

* * * * *

EXHIBIT 45



US010604788B2

(12) **United States Patent**
Wu et al.

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(45) **Date of Patent:** **Mar. 31, 2020**

(54) **SYSTEM FOR PROCESSING
POLYNUCLEOTIDE-CONTAINING SAMPLES**

(58) **Field of Classification Search**

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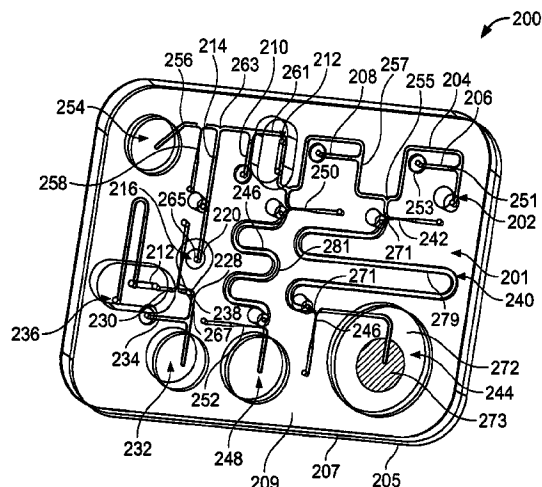
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(57) **ABSTRACT**

Methods and systems for processing polynucleotides (e.g.,
DNA) are disclosed. A processing region includes one or
more surfaces (e.g., particle surfaces) modified with ligands
that retain polynucleotides under a first set of conditions
(e.g., temperature and pH) and release the polynucleotides
under a second set of conditions (e.g., higher temperature
and/or more basic pH). The processing region can be used
to, for example, concentrate polynucleotides of a sample
and/or separate inhibitors of amplification reactions from the
polynucleotides. Microfluidic devices with a processing
region are disclosed.

55 Claims, 25 Drawing Sheets



US 10,604,788 B2

Page 2

Related U.S. Application Data

- continuation of application No. 14/506,471, filed on Oct. 3, 2014, now Pat. No. 10,364,456, which is a continuation of application No. 11/281,247, filed on Nov. 16, 2005, now Pat. No. 8,852,862, which is a continuation-in-part of application No. PCT/US2005/015345, filed on May 3, 2005.
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CPC **B01F 13/0059** (2013.01); **B01L 3/50273** (2013.01); **B01L 3/502707** (2013.01); **B01L 3/502738** (2013.01); **B01L 3/502753** (2013.01); **B01L 3/523** (2013.01); **C12N 15/101** (2013.01); **C12N 15/1006** (2013.01); **C12Q 1/686** (2013.01); **F16K 99/0001** (2013.01); **F16K 99/0019** (2013.01); **F16K 99/0032** (2013.01); **F16K 99/0036** (2013.01); **F16K 99/0044** (2013.01); **B01L 3/5082** (2013.01); **B01L 3/502715** (2013.01); **B01L 3/502723** (2013.01); **B01L 3/565** (2013.01); **B01L 7/52** (2013.01); **B01L 2200/10** (2013.01); **B01L 2200/16** (2013.01); **B01L 2300/0672** (2013.01); **B01L 2300/087** (2013.01); **B01L 2300/0816** (2013.01); **B01L 2300/0867** (2013.01); **B01L 2300/0887** (2013.01); **B01L 2400/0442** (2013.01); **B01L 2400/0481** (2013.01); **B01L 2400/0487** (2013.01); **B01L 2400/0633** (2013.01); **B01L 2400/0677** (2013.01); **B01L 2400/0683** (2013.01); **B01L 2400/0694** (2013.01); **F16K 99/0034** (2013.01); **F16K 2099/0084** (2013.01)
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US 10,604,788 B2

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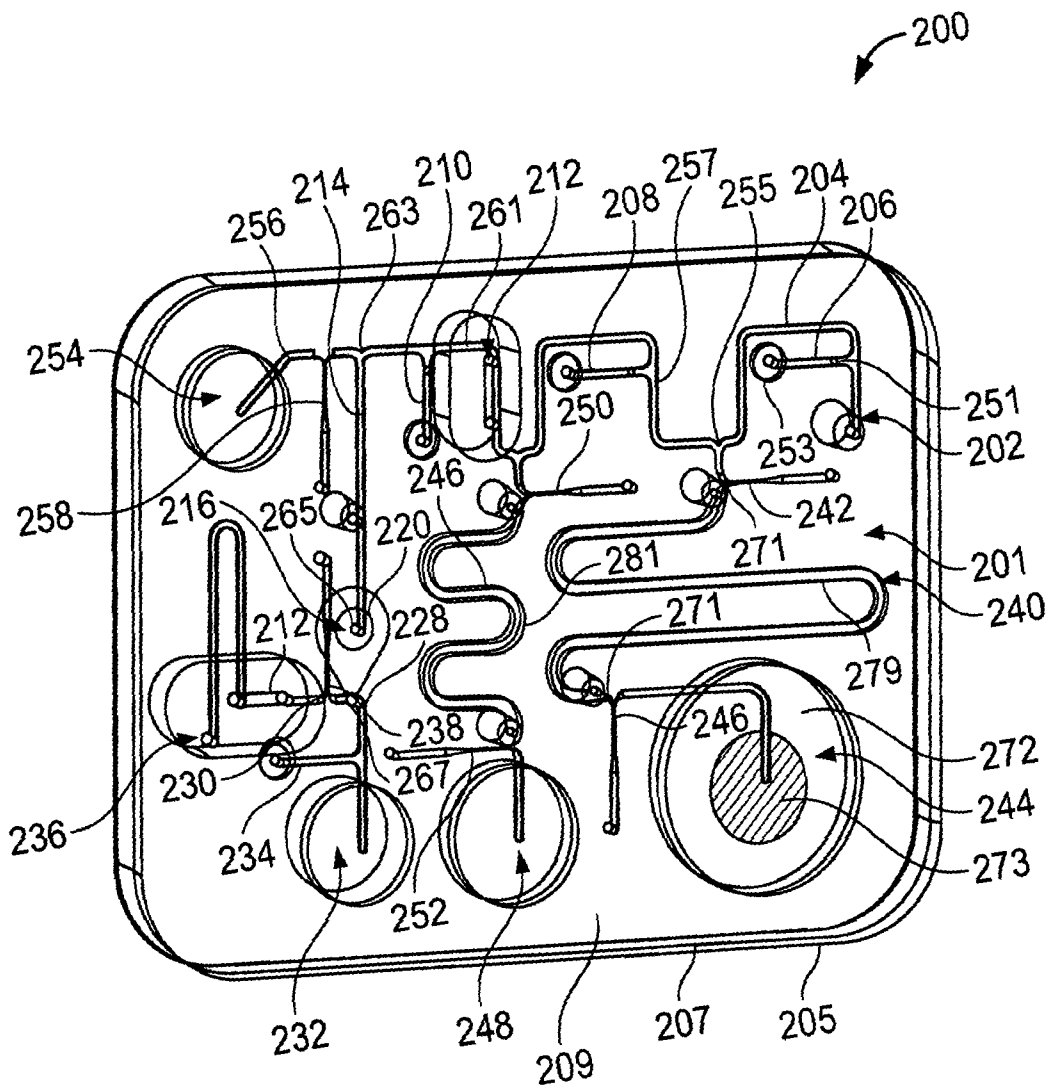


FIG. 1

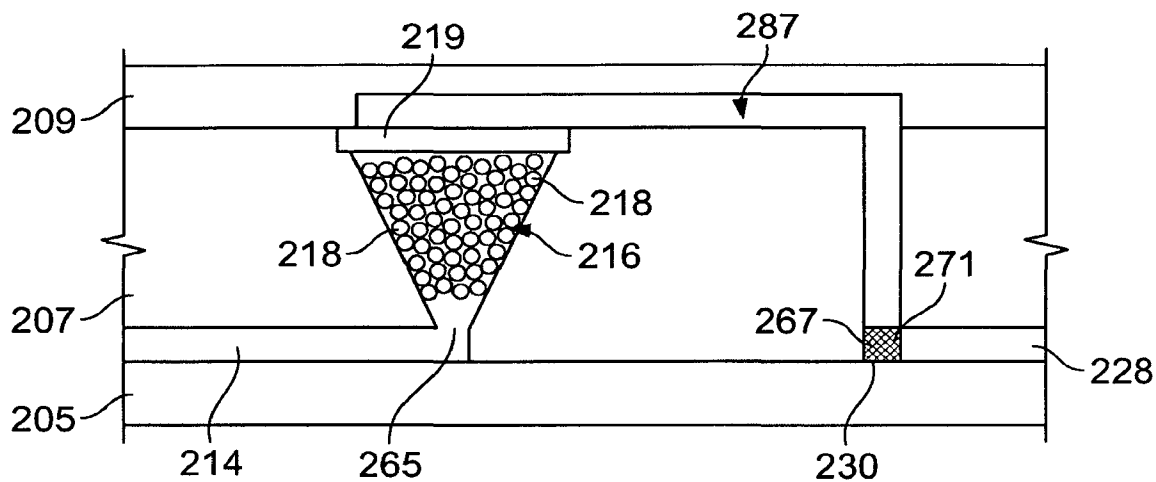


FIG. 2

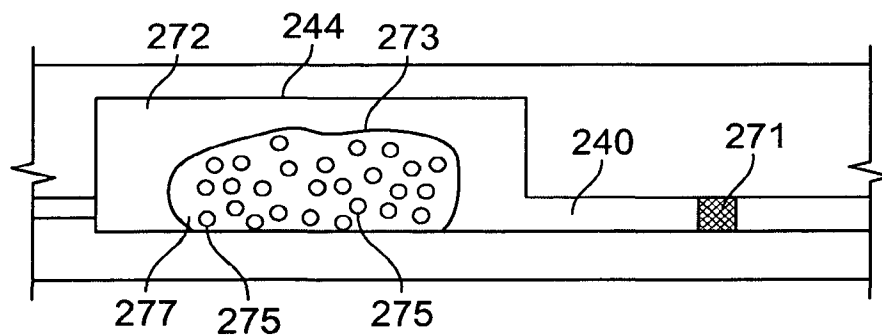


FIG. 3

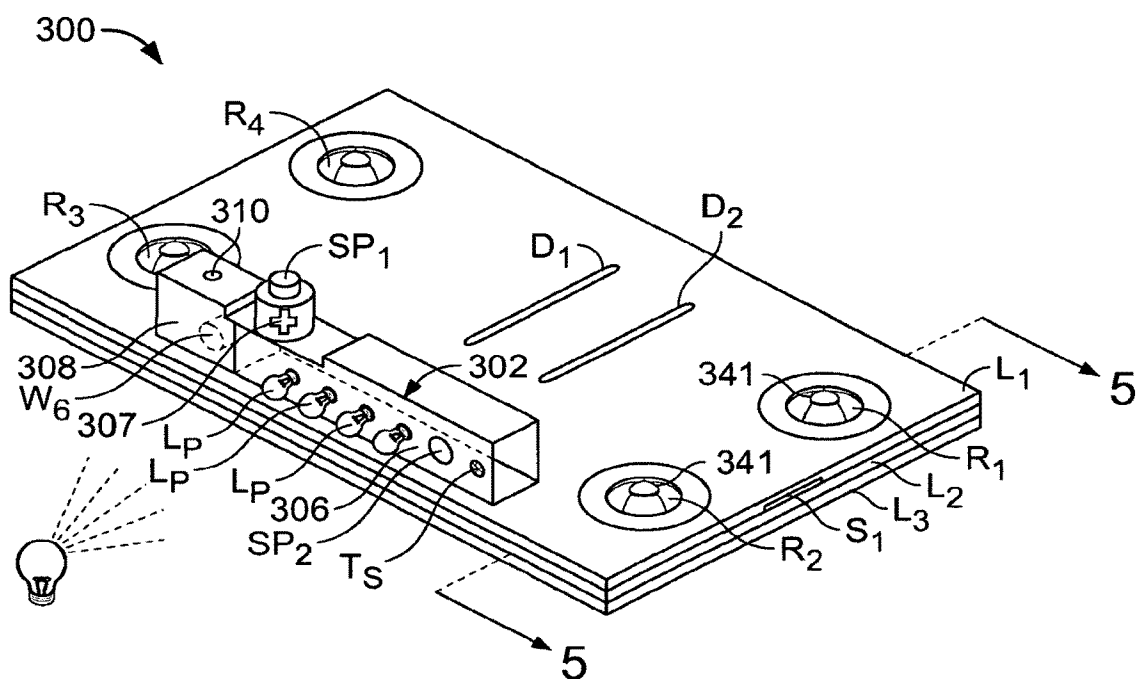


FIG. 4

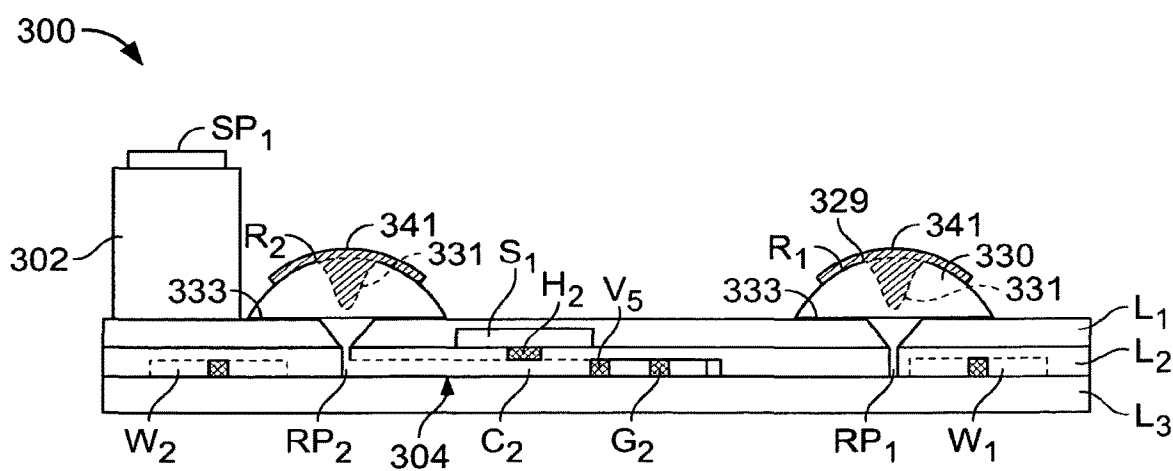


FIG. 5

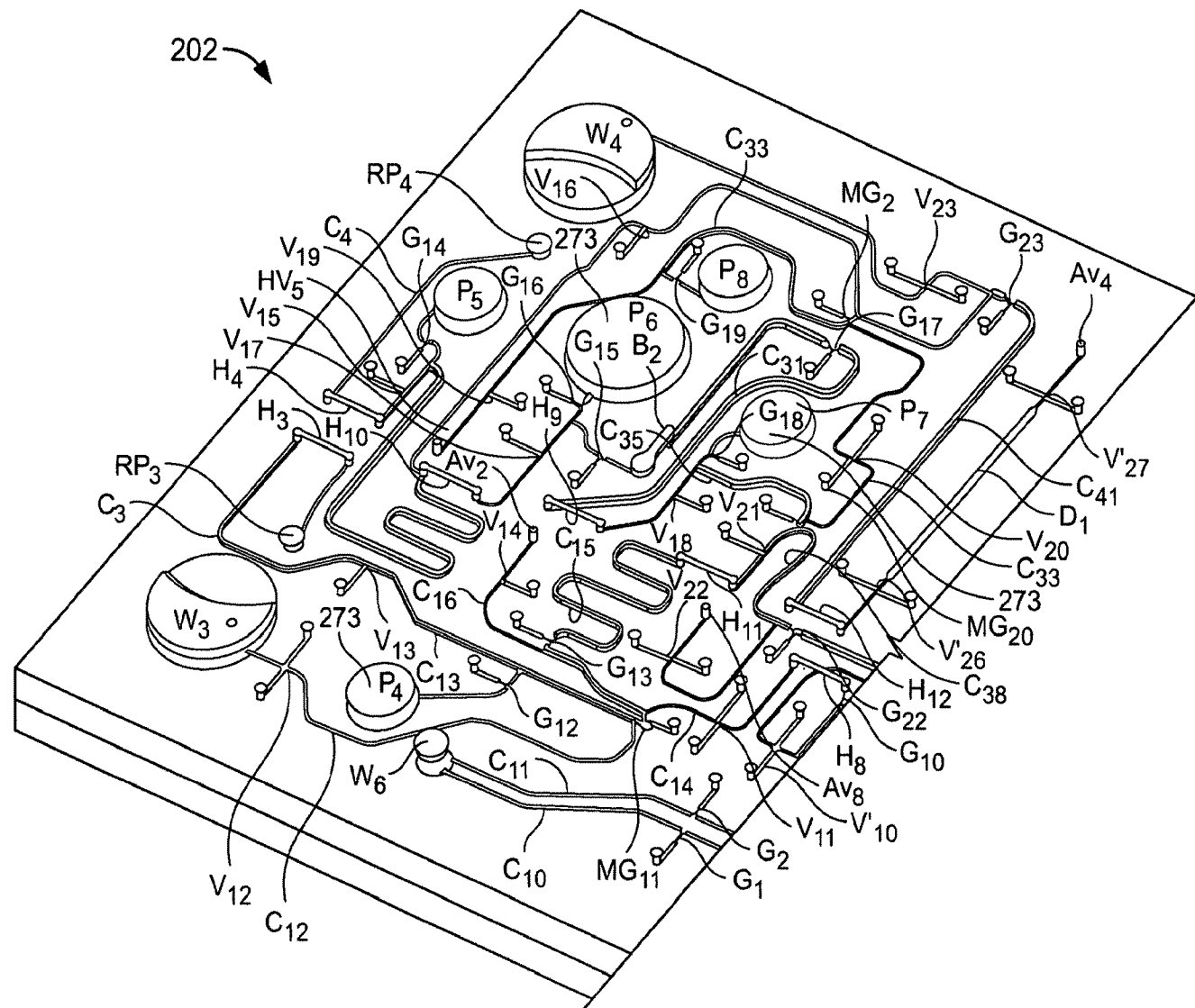


FIG. 6A

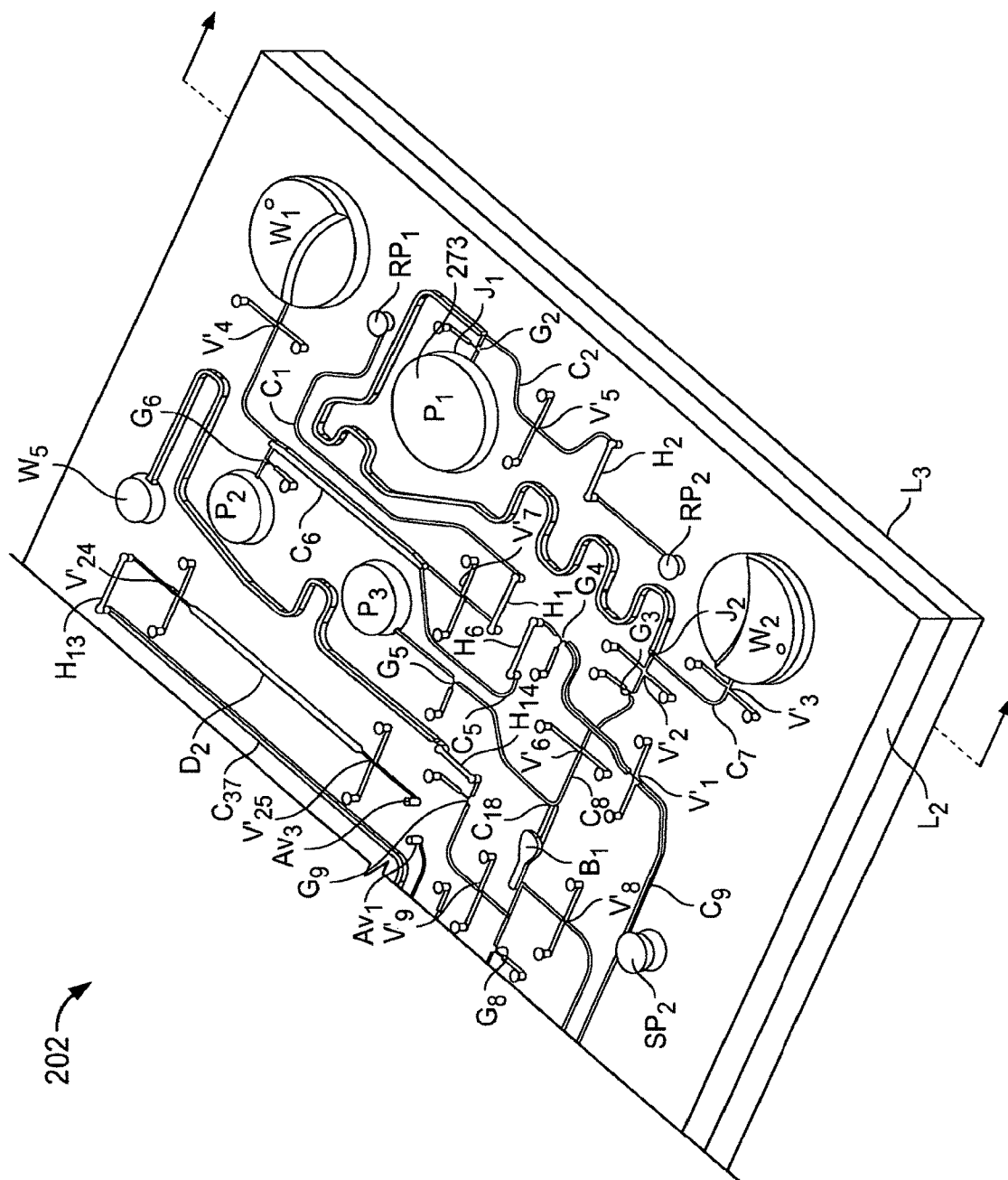


FIG. 6B

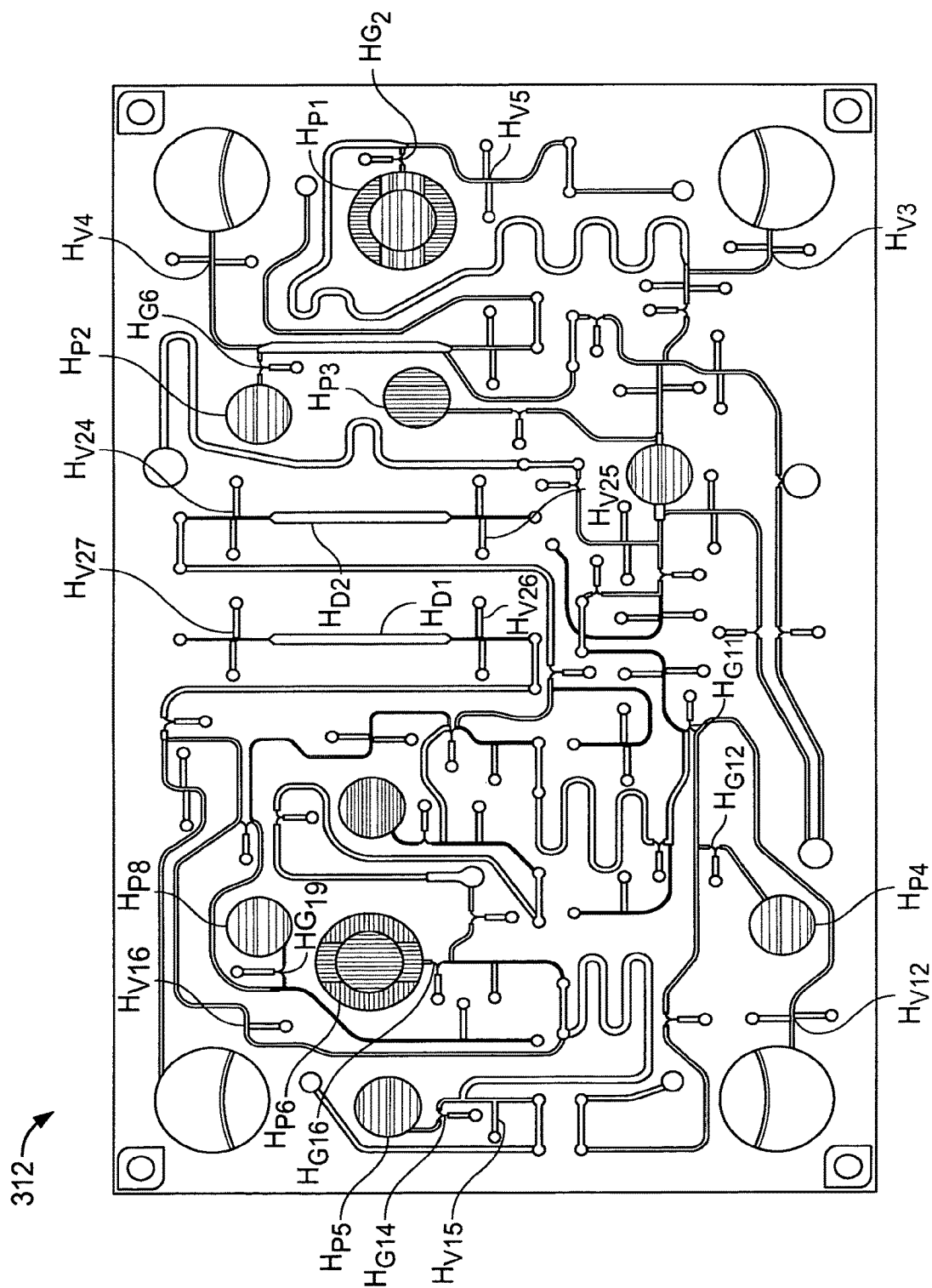


FIG. 7

U.S. Patent

Mar. 31, 2020

Sheet 7 of 25

US 10,604,788 B2

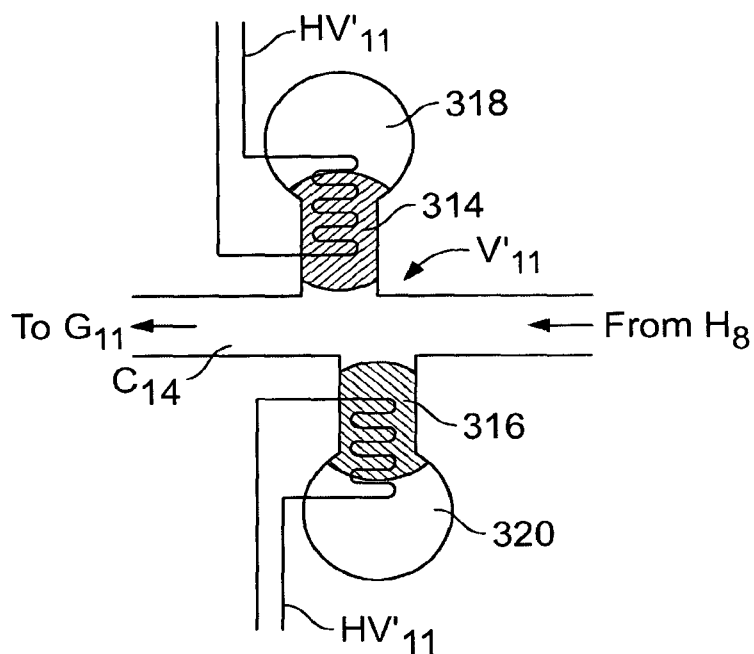


FIG. 8

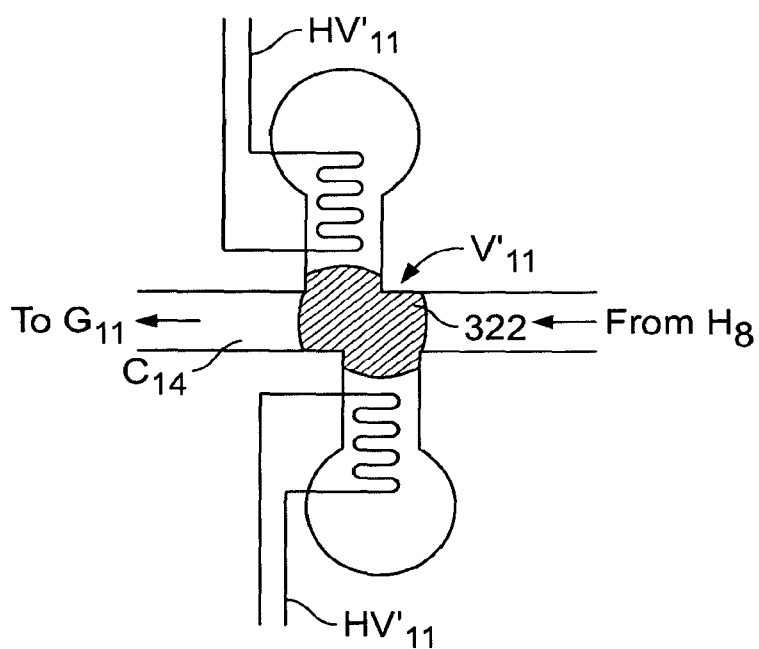


FIG. 9

U.S. Patent

Mar. 31, 2020

Sheet 8 of 25

US 10,604,788 B2

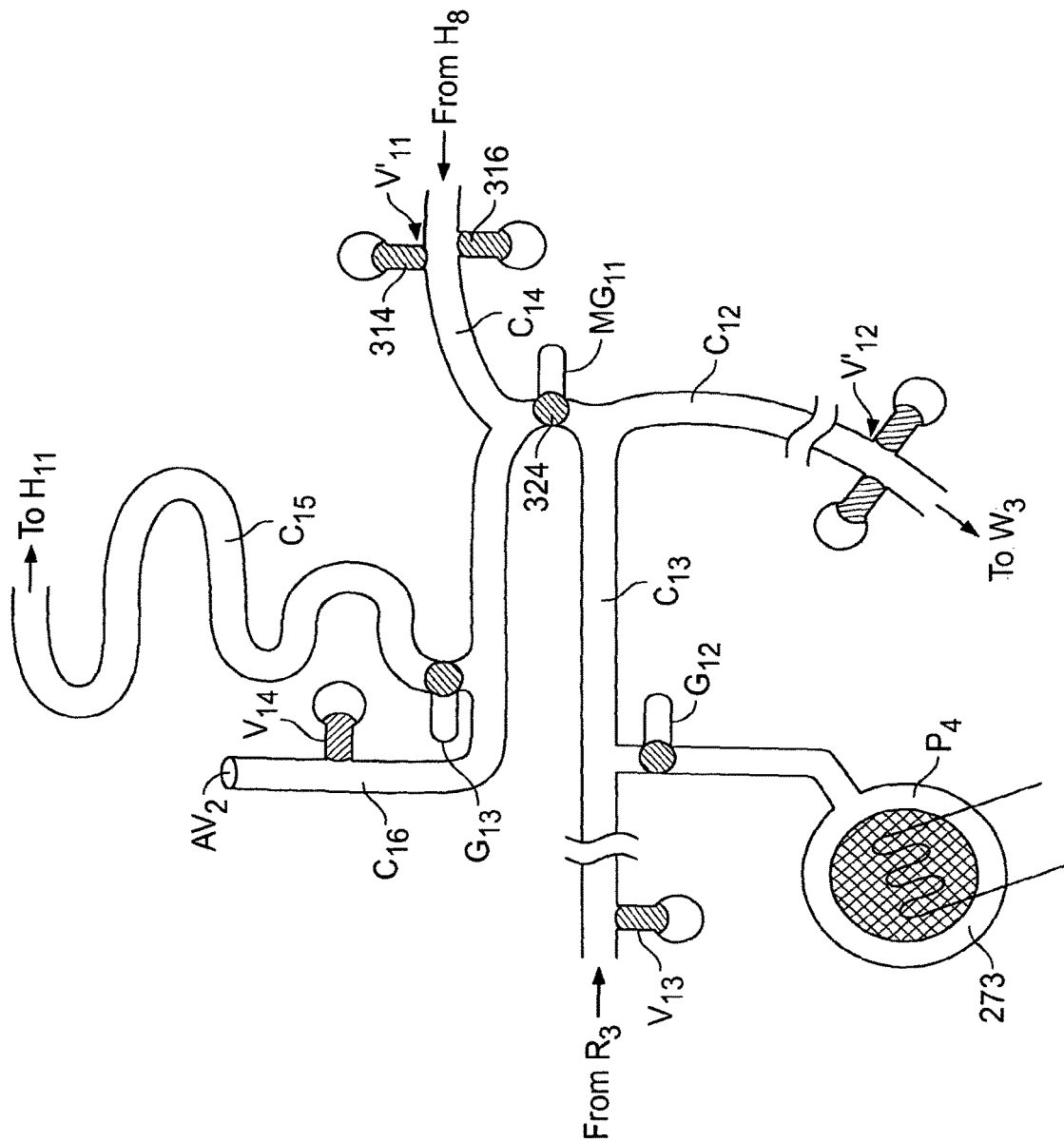


FIG. 10A

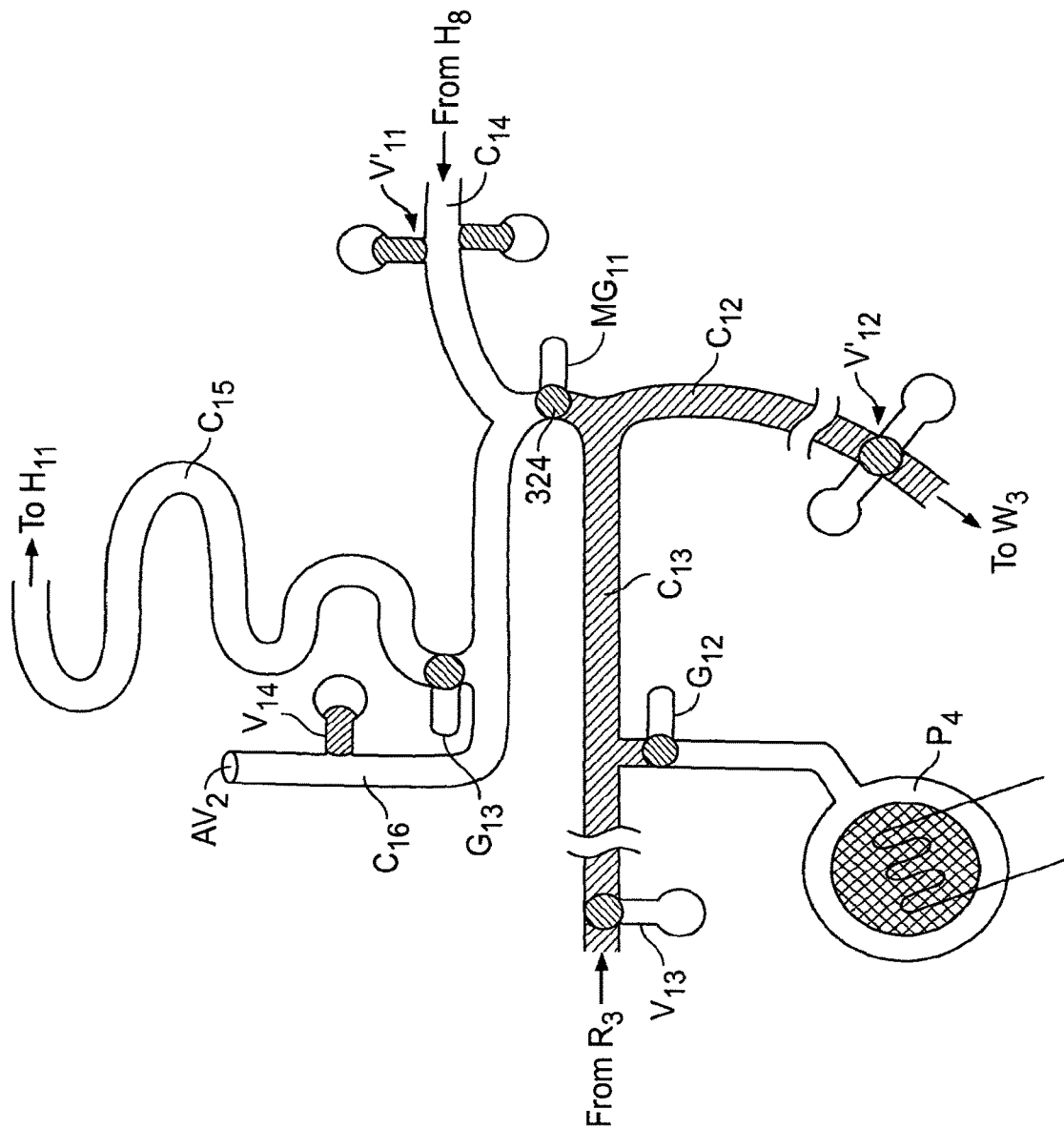


FIG. 10B

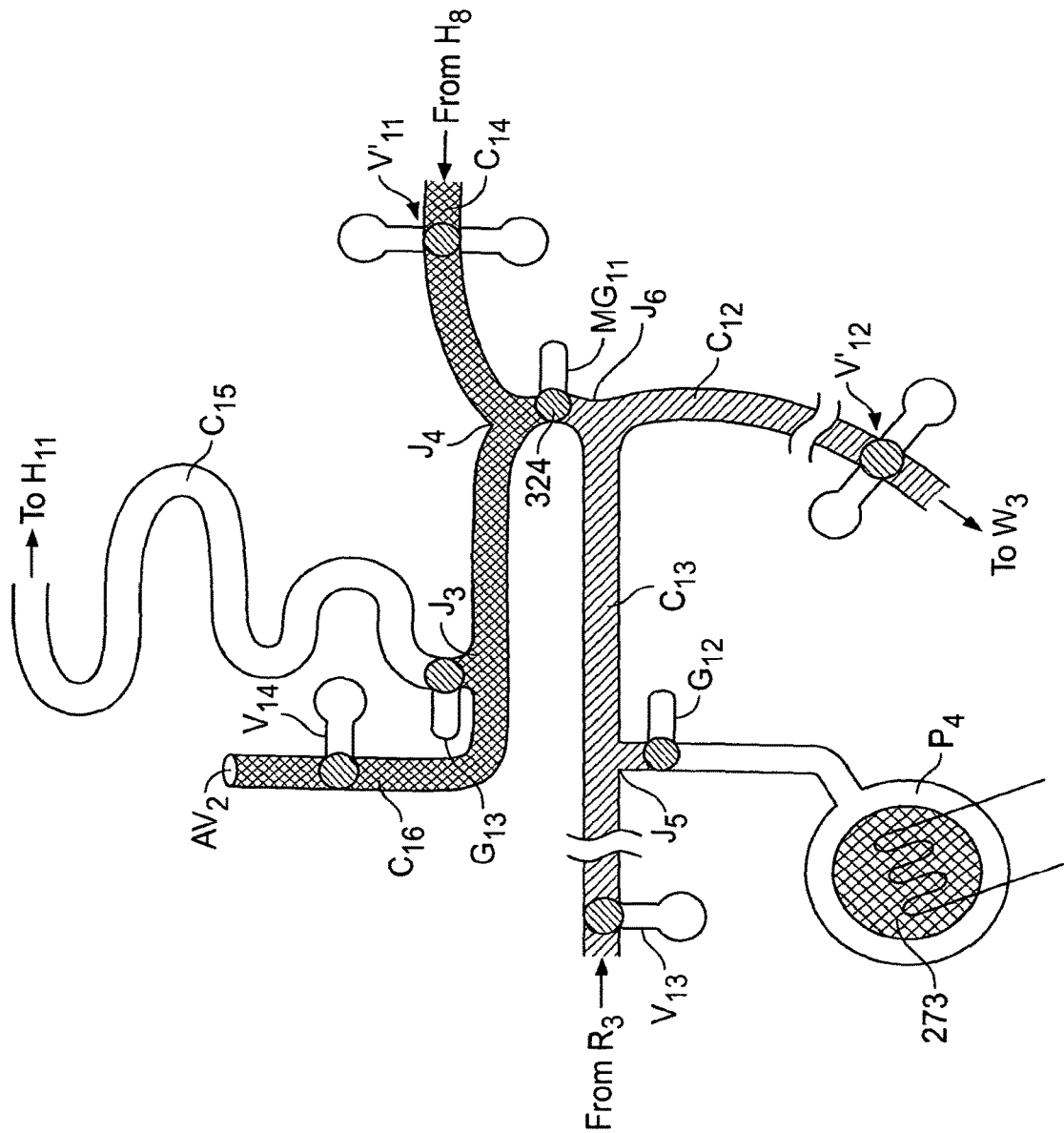


FIG. 10C

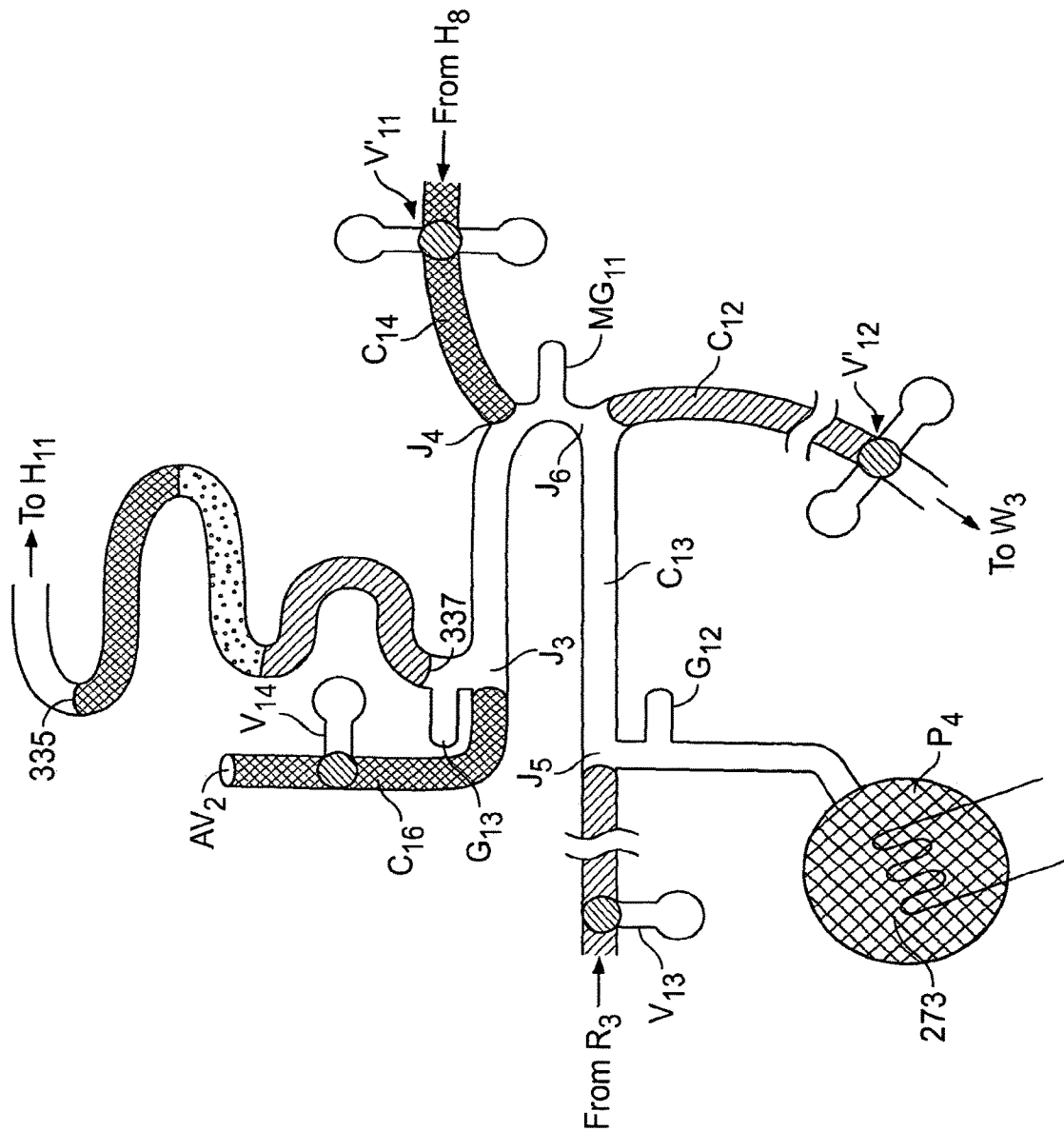


FIG. 10D

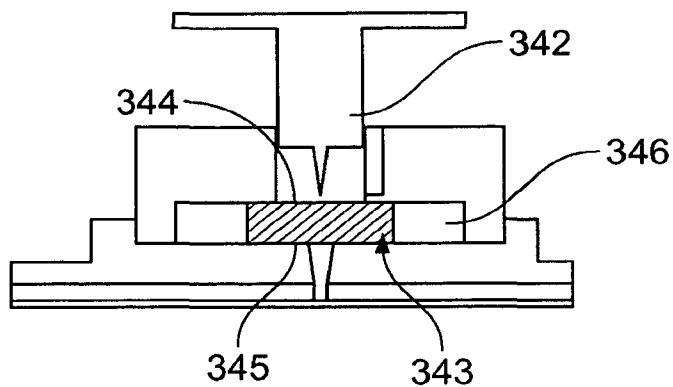


FIG. 11A

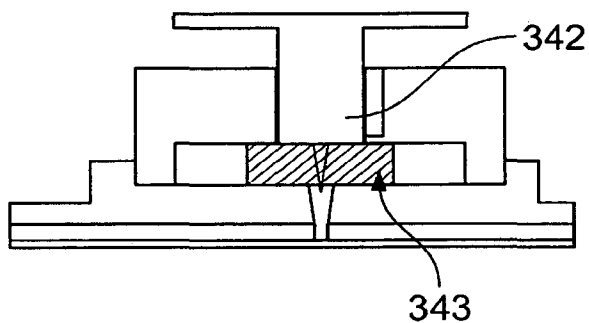


FIG. 11B

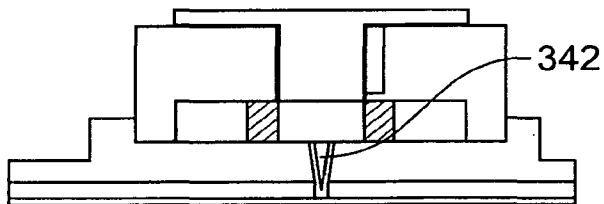


FIG. 11C

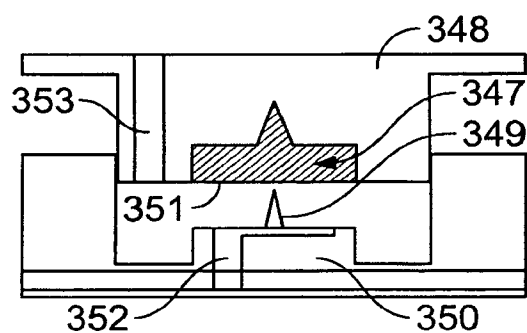


FIG. 12A

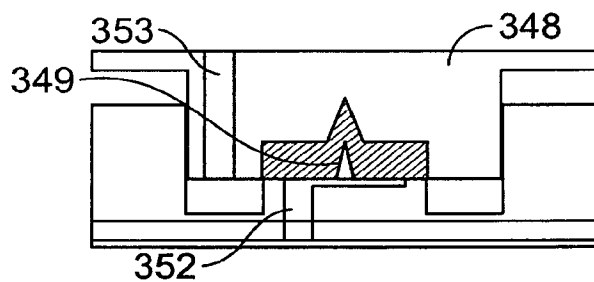


FIG. 12B

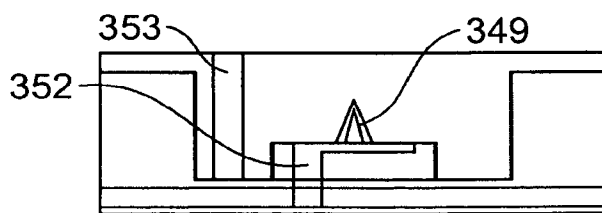


FIG. 12C

U.S. Patent

Mar. 31, 2020

Sheet 14 of 25

US 10,604,788 B2

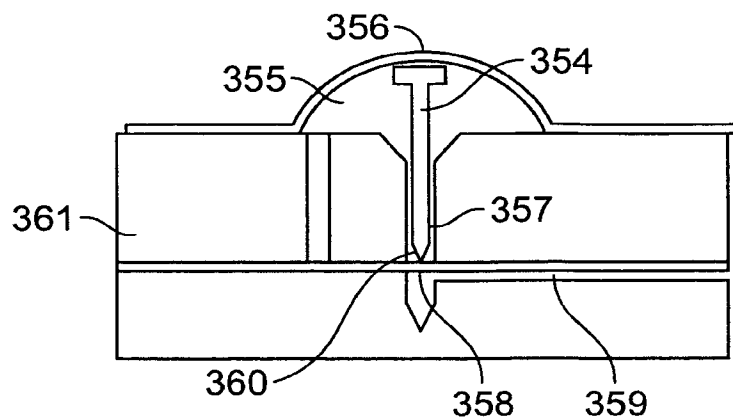


FIG. 13

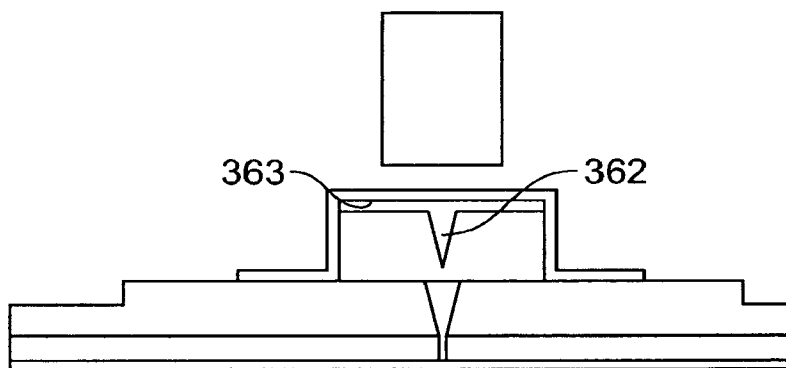


FIG. 14A

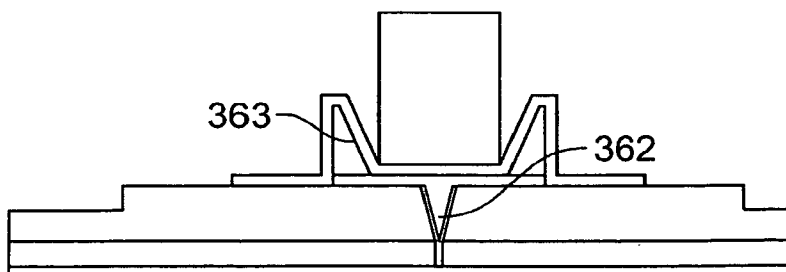


FIG. 14B

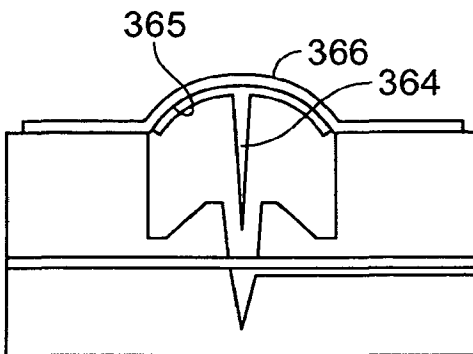


FIG. 15A

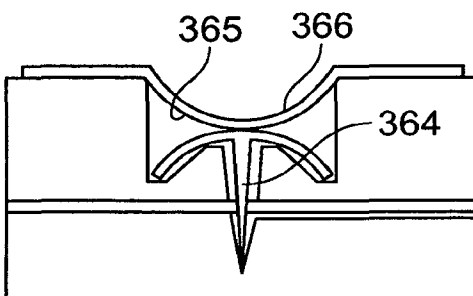


FIG. 15B

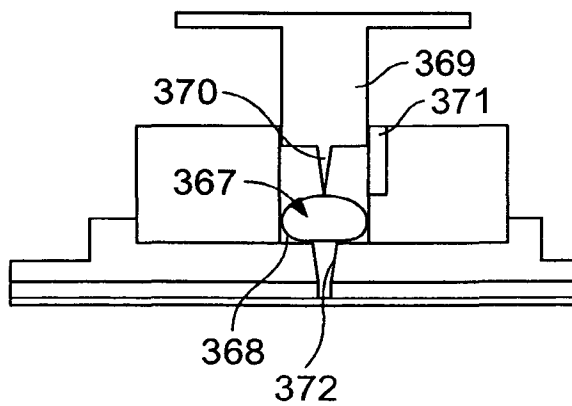


FIG. 16

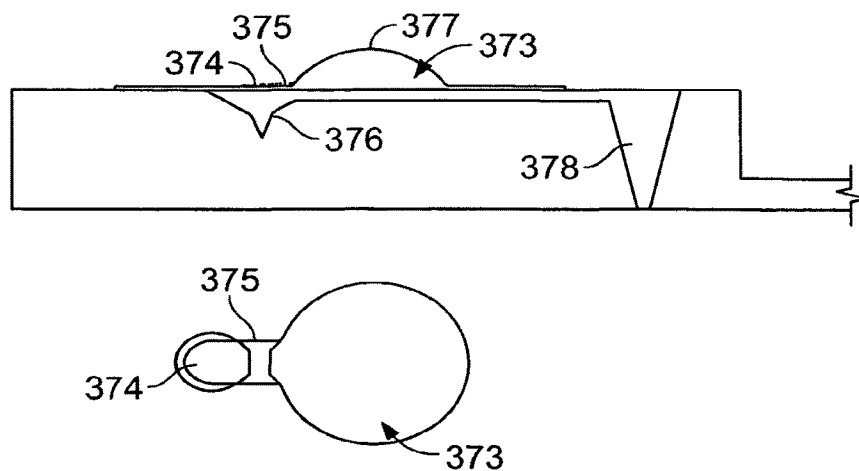


FIG. 17

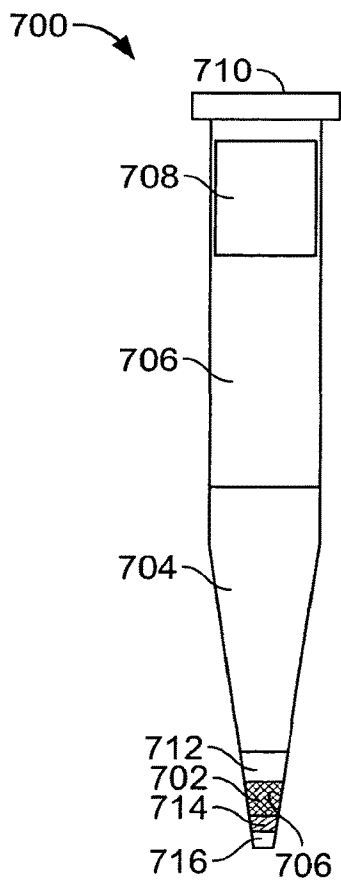


FIG. 18

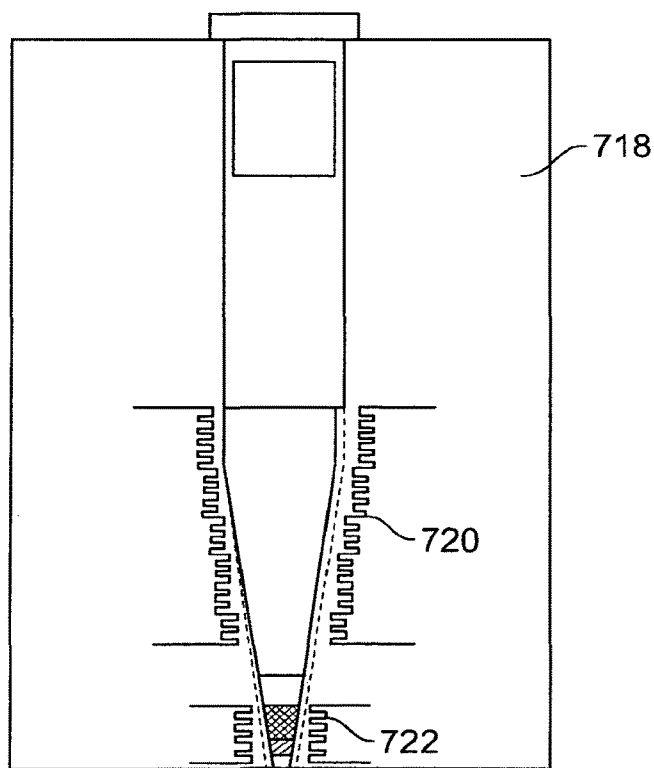


FIG. 19

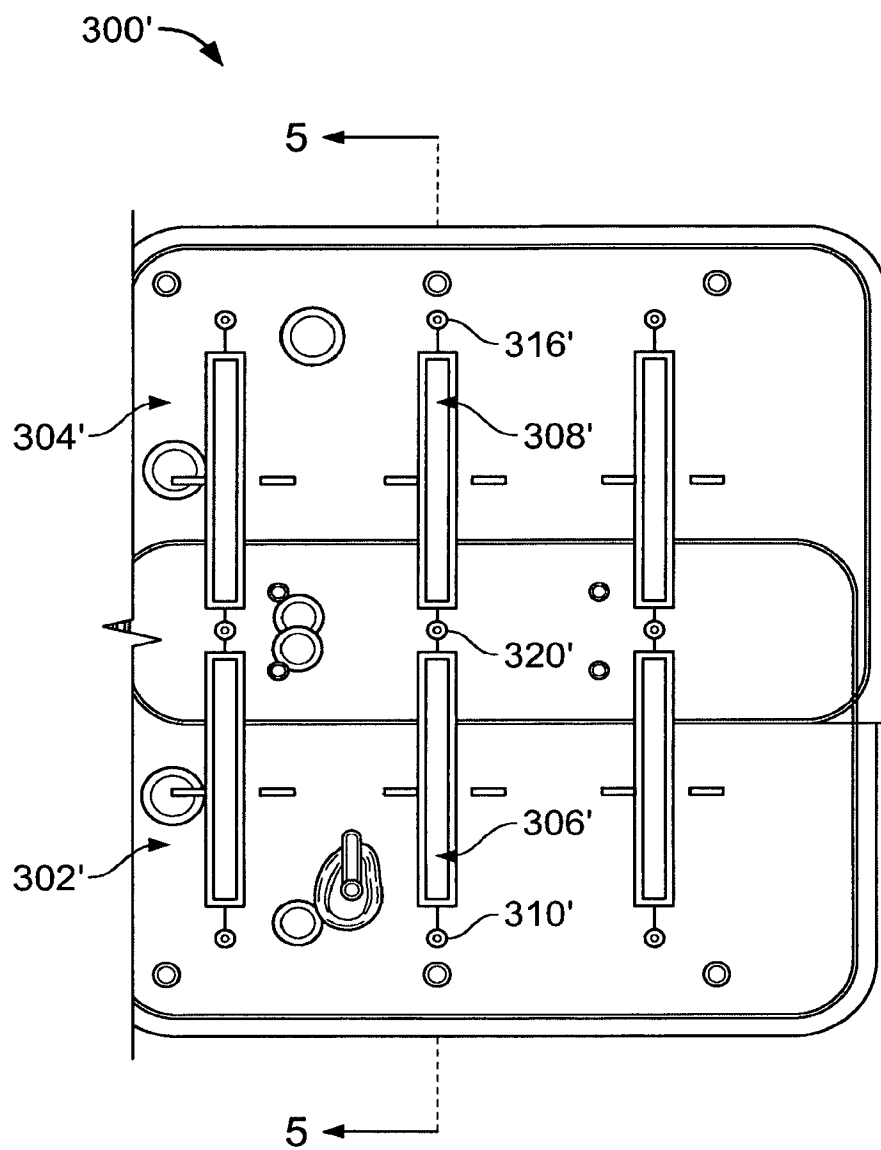


FIG. 20

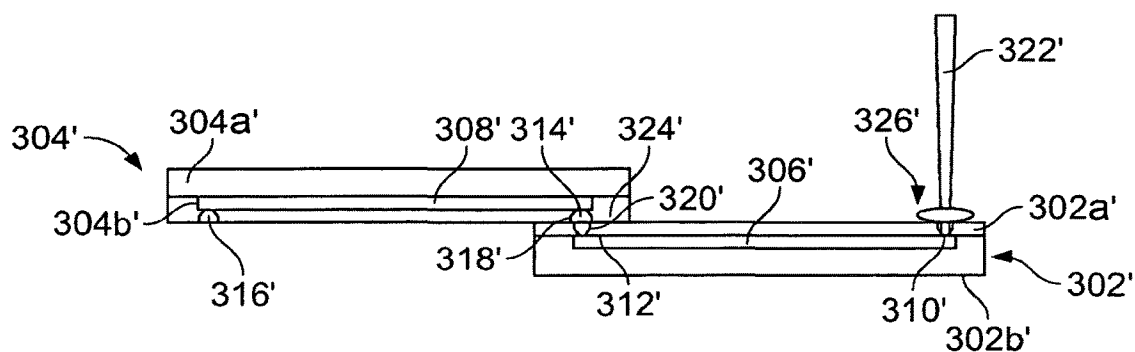


FIG. 21

DNA Capture by Poly-L-Lysine Beads

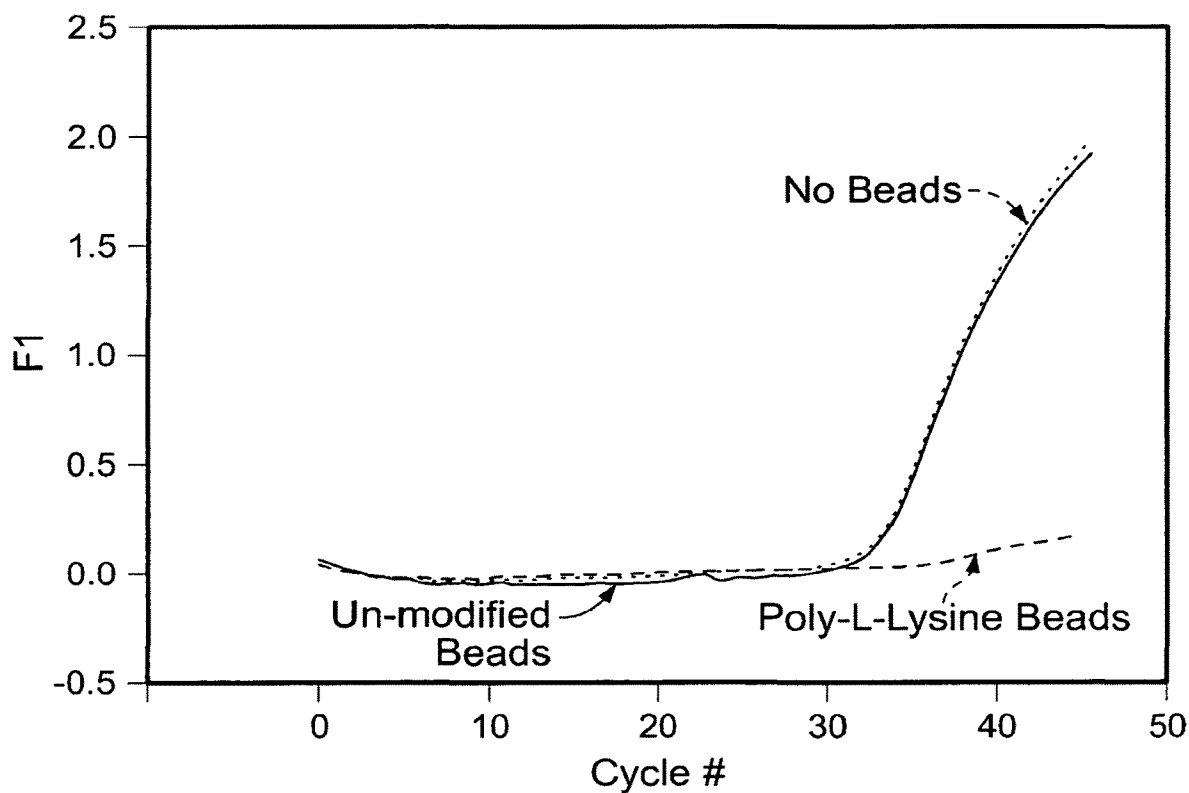


FIG. 22

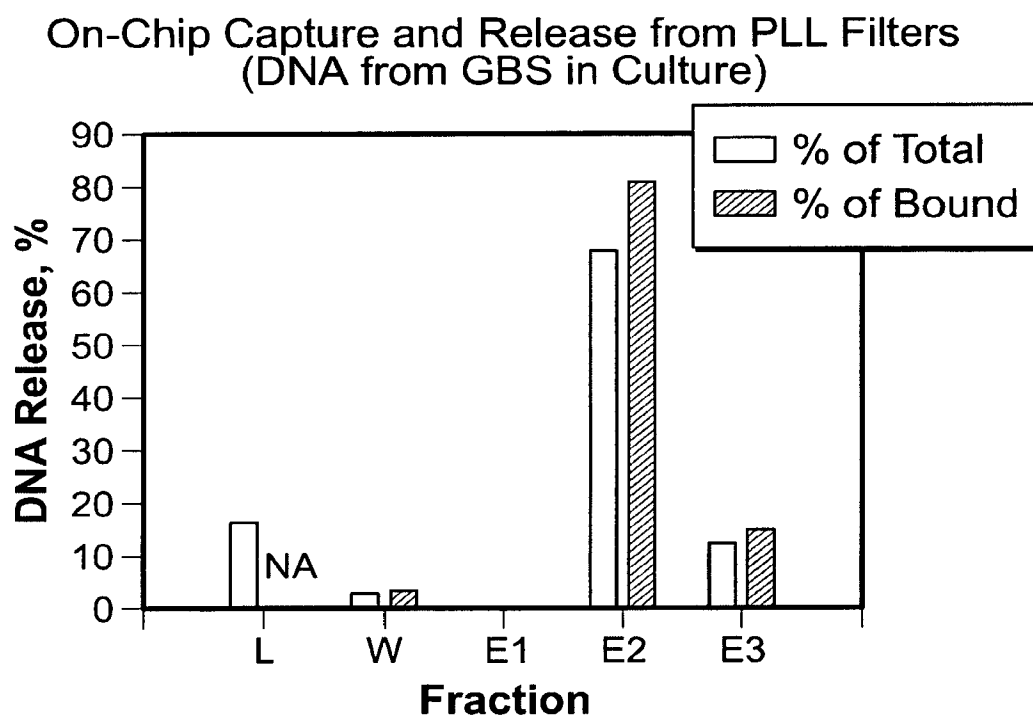


FIG. 23

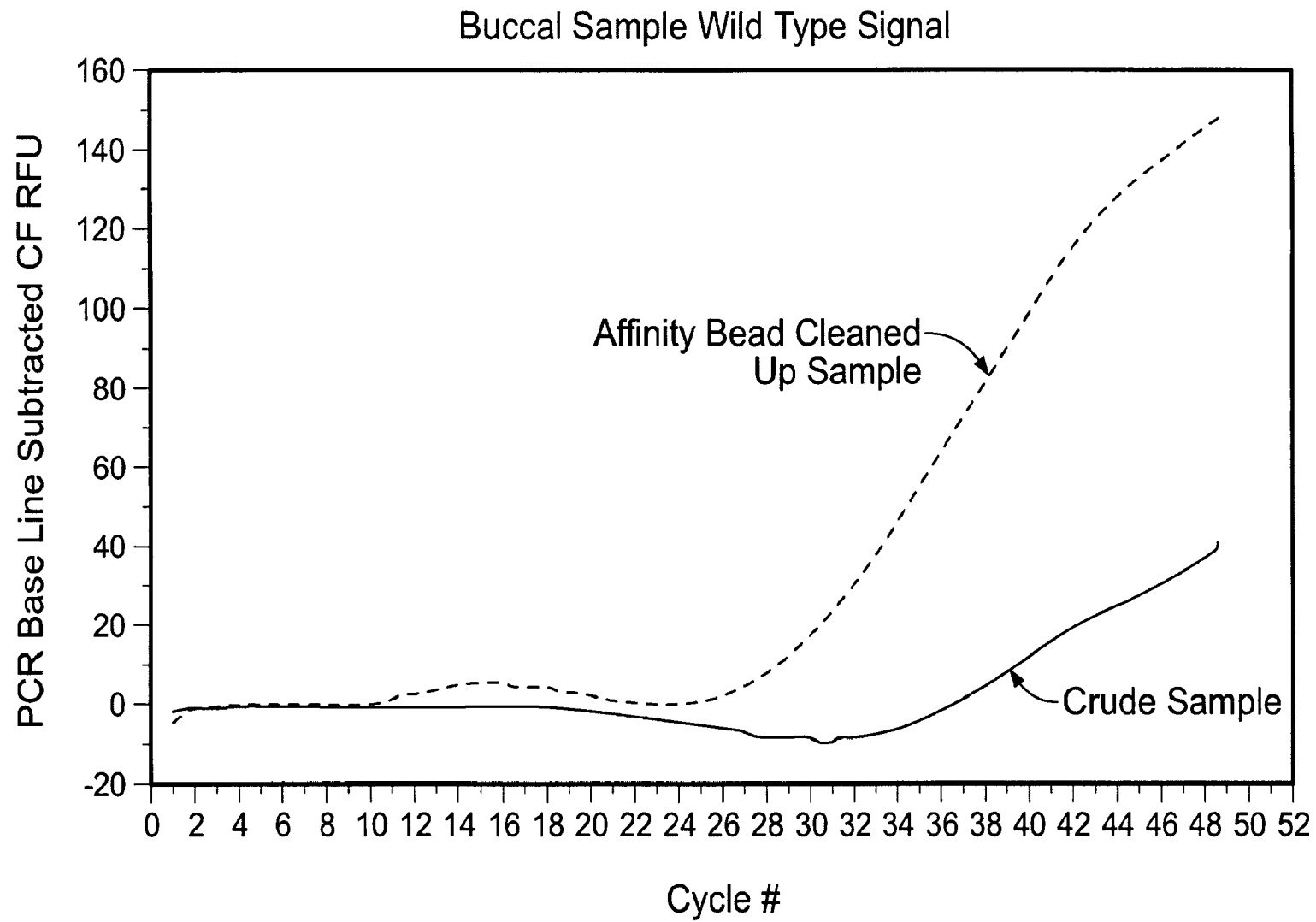


FIG. 24

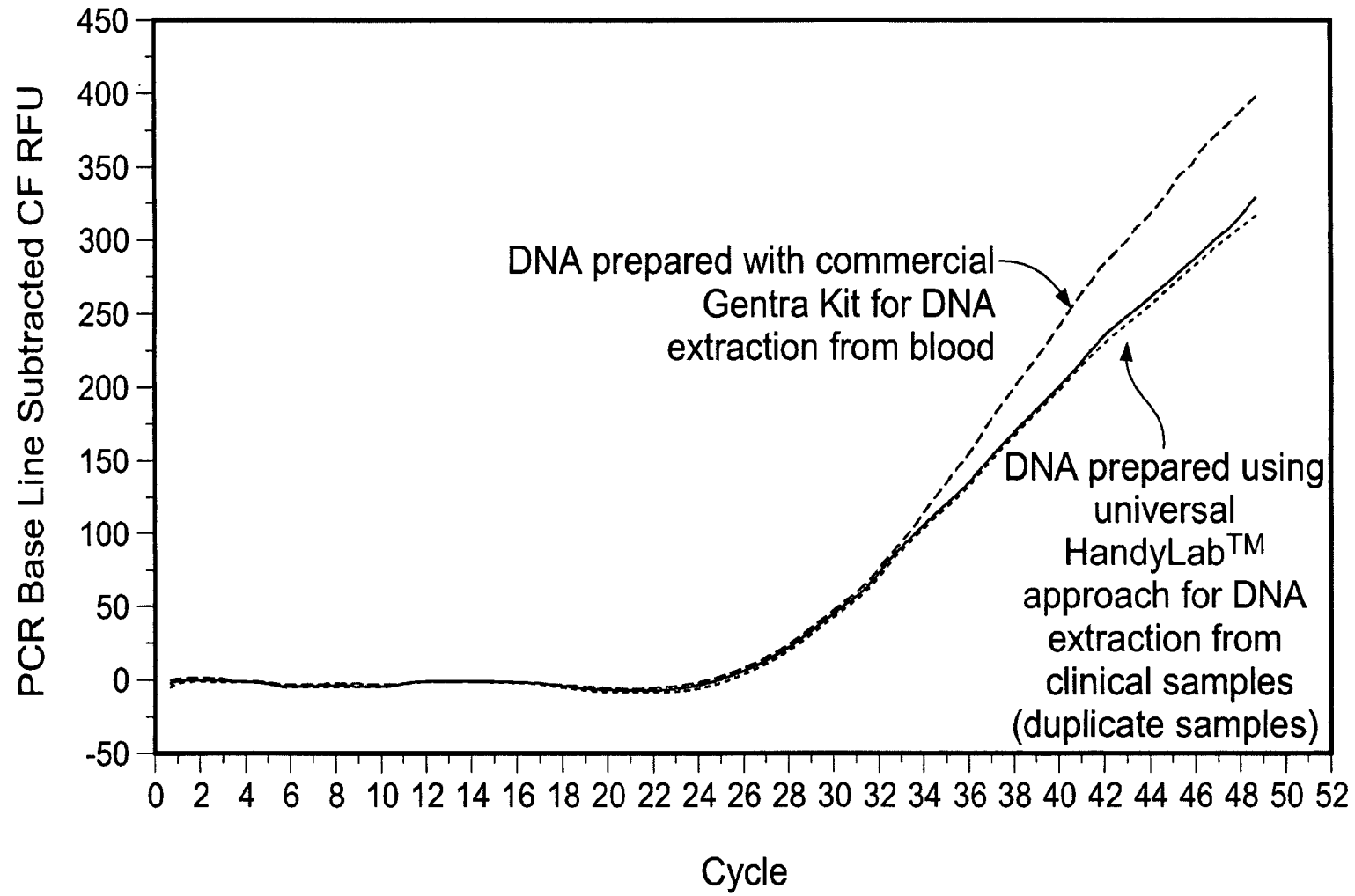


FIG. 25

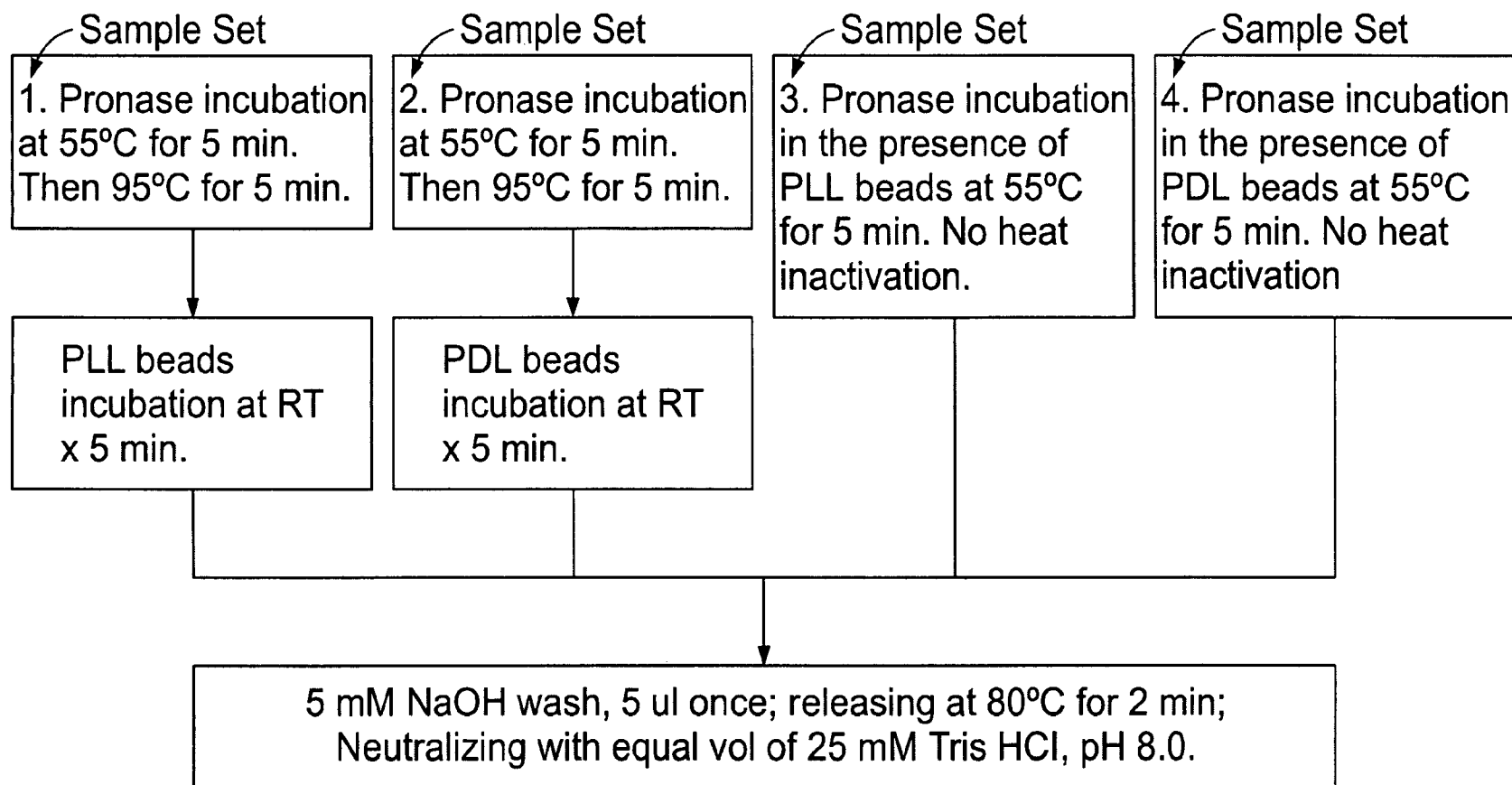


FIG. 26A

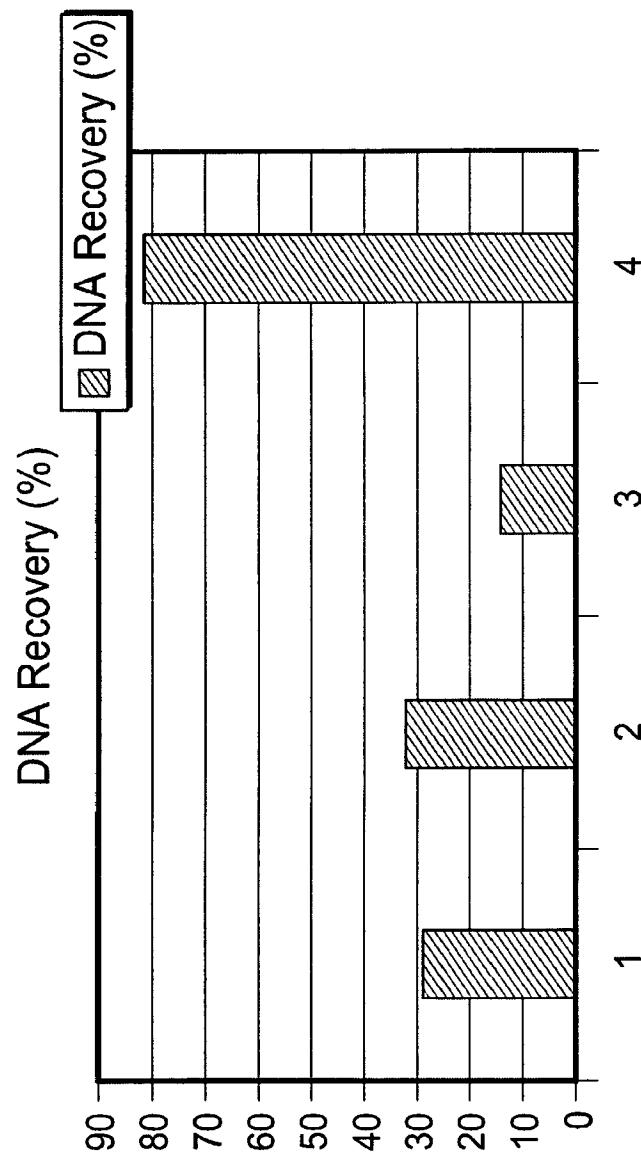


FIG. 26B

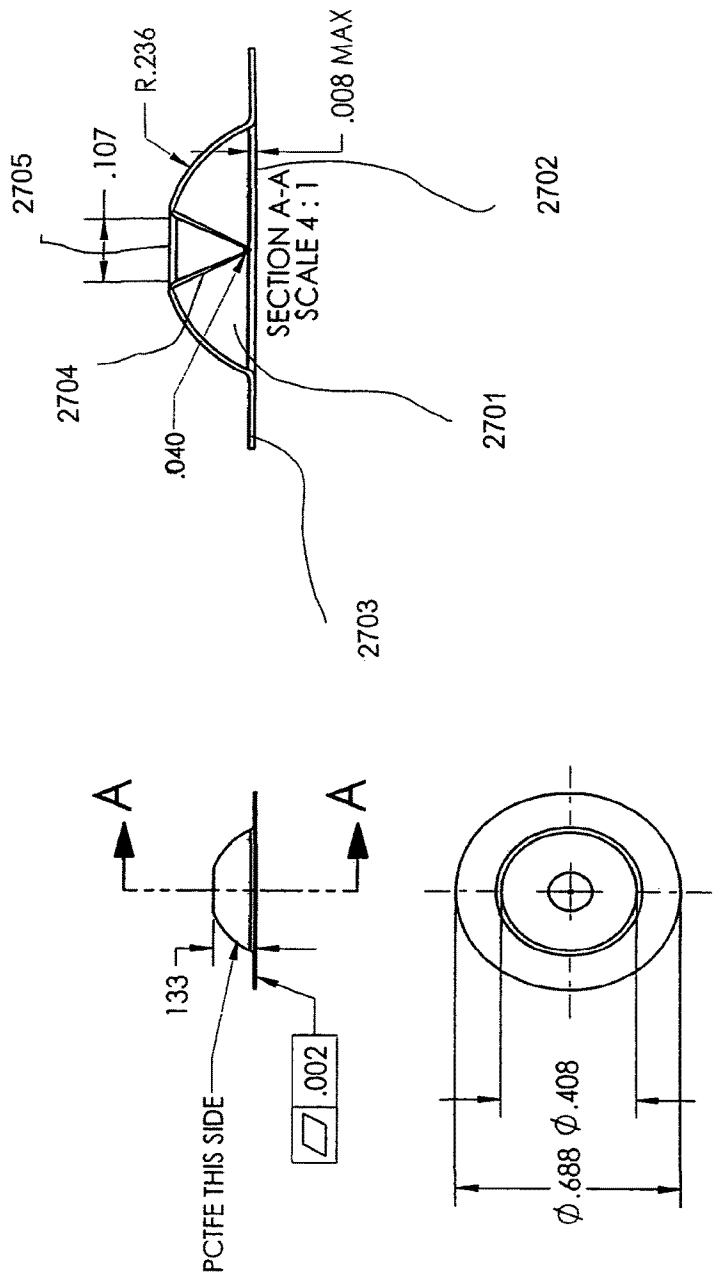


FIG. 27A

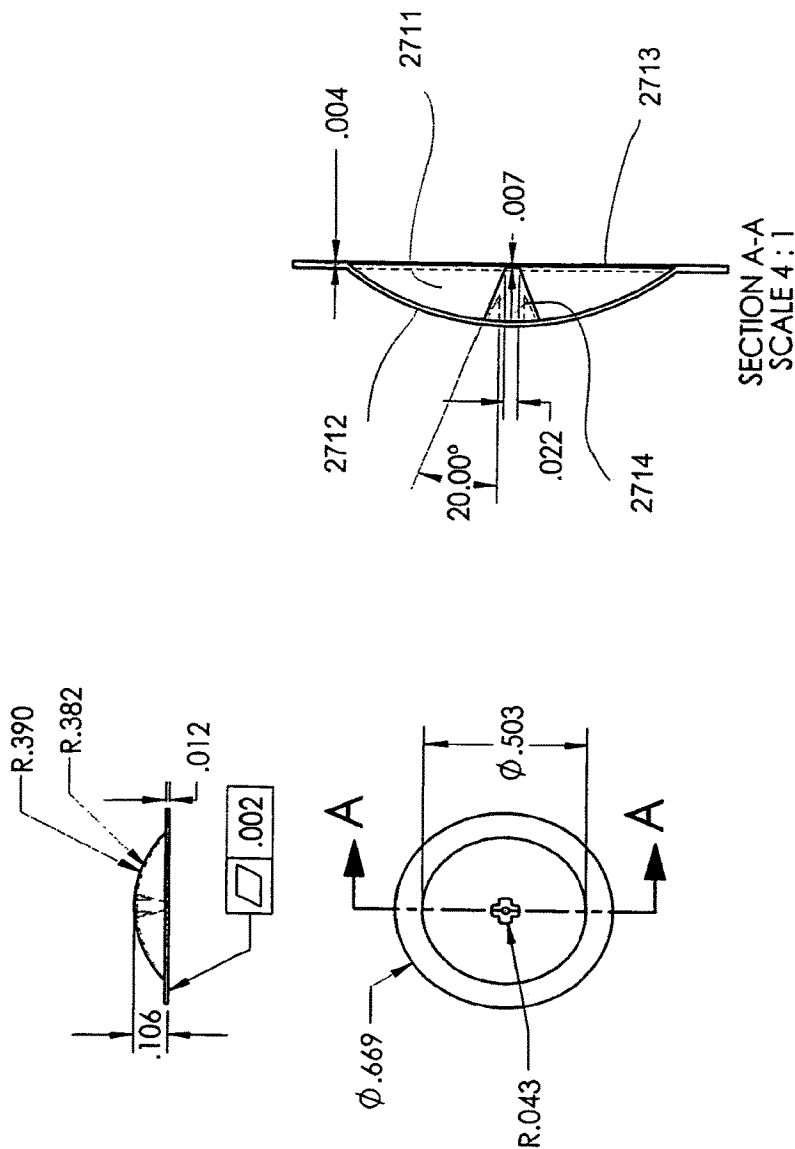


FIG. 27B

US 10,604,788 B2

1

SYSTEM FOR PROCESSING POLYNUCLEOTIDE-CONTAINING SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 16/518,735, filed Jul. 22, 2019, which is a continuation of U.S. patent application Ser. No. 14/506,471, filed Oct. 3, 2014 and issued as U.S. Pat. No. 10,364,456 on Jul. 30, 2019, which is a continuation of U.S. patent application Ser. No. 11/281,247, filed Nov. 16, 2005 and issued as U.S. Pat. No. 8,852,862 on Oct. 7, 2014, which is a continuation-in-part of International Application No. PCT/US2005/015345, filed May 3, 2005, which claims the benefit of priority of U.S. Provisional Application No. 60/567,174, filed May 3, 2004, and U.S. Provisional Application No. 60/645,784, filed Jan. 21, 2005. Each of these applications is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods for processing polynucleotide-containing samples as well as to related systems.

BACKGROUND

The analysis of a biological sample often includes detecting one or more polynucleotides present in the sample. One example of detection is qualitative detection, which relates, for example, to the determination of the presence of the polynucleotide and/or the determination of information related to, for example, the type, size, presence or absence of mutations, and/or the sequence of the polynucleotide. Another example of detection is quantitative detection, which relates, for example, to the determination of the amount of polynucleotide present. Detection may include both qualitative and quantitative aspects.

Detecting polynucleotides often involves the use of an enzyme. For example, some detection methods include polynucleotide amplification by polymerase chain reaction (PCR) or a related amplification technique. Other detection methods that do not amplify the polynucleotide to be detected also make use of enzymes. However, the functioning of enzymes used in such techniques may be inhibited by the presence of inhibitors present along with the polynucleotide to be detected. The inhibitors may interfere with, for example, the efficiency and/or specificity of the enzymes.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a method and related systems for processing one or more polynucleotides (e.g., to concentrate the polynucleotide(s) and/or to separate the polynucleotide(s) from inhibitor compounds (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucosal compounds, DNA binding proteins, or a saccharide) that might inhibit detection and/or amplification of the polynucleotides).

In some embodiments, the method includes contacting the polynucleotides and a relatively immobilized compound that preferentially associates with (e.g., retains) the polynucleotides as opposed to inhibitors. An exemplary compound is a poly-cationic polyamide (e.g., poly-L-lysine and/or poly-D-lysine), or polyethyleneimine (PEI), which may be bound to a surface (e.g., a surface of one or more particles). The

2

compound retains the polynucleotides so that the polynucleotides and inhibitors may be separated, such as by washing the surface with the compound and associated polynucleotides. Upon separation, the association between the polynucleotide and compound may be disrupted to release (e.g., separate) the polynucleotides from the compound and surface.

In some embodiments, the surface (e.g., a surface of one or more particles) is modified with a poly-cationic substance such as a polyamide or PEI, which may be covalently bound to the surface. The poly-cationic polyamide may include at least one of poly-L-lysine and poly-D-lysine. In some embodiments, the poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) have an average molecular weight of at least about 7500 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have an average molecular weight of less than about 35,000 Da (e.g., an average molecular weight of less than about 30000 Da (e.g., an average molecular weight of about 25,000 Da)). The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of at least about 15,000 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of less than about 25,000 Da (e.g., a median molecular weight of less than about 20,000 Da (e.g., a median molecular weight of about 20,000 Da)). If the polycationic material is PEI, its molecular weight is preferably in the range 600-800 Daltons.

Another aspect of the invention relates to a sample preparation device including a surface including a polycationic polyamide or PEI bound thereto and a sample introduction passage in communication with the surface for contacting the surface with a fluidic sample.

In some embodiments, the device includes a heat source configured to heat an aqueous liquid in contact with the surface to at least about 65° C.

In some embodiments, the device includes a reservoir of liquid having a pH of at least about 10 (e.g., about 10.5 or more). The device is configured to contact the surface with the liquid (e.g., by actuating a pressure source to move the liquid).

In some embodiments, the surface comprises surfaces of a plurality of particles.

In some embodiments, the poly-cationic polyamide includes poly-L-lysine and/or poly-D-lysine.

Another aspect of the invention relates to a method for processing a sample including providing a mixture including a liquid and an amount of polynucleotide, contacting a retention member with the mixture. The retention member may be configured to preferentially retain polynucleotides as compared to polymerase chain reaction inhibitors. Substantially all of the liquid in the mixture is removed from the retention member. The polynucleotides are released from the retention member. The polynucleotide may have a size of less than about 7.5 Mbp.

The liquid may be a first liquid and removing substantially all of the liquid from the retention member may include contacting the retention member with a second liquid.

Contacting the retention member with a second liquid can include actuating a thermally actuated pressure source to apply a pressure to the second liquid. Contacting the retention member with a second liquid can include opening a thermally actuated valve to place the second liquid in fluid communication with the retention member.

US 10,604,788 B2

3

The second liquid may have a volume of less than about 50 microliters.

The retention member may include a surface having a compound configured to bind polynucleotides preferentially to polymerase chain reaction inhibitors (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucousol compounds, DNA binding proteins, or a saccharide).

The surface may include a poly-lysine (e.g., poly-L-lysine and/or poly-D-lysine) or PEI.

The second liquid may include a detergent (e.g., SDS).

Releasing may include heating the retention member to a temperature of at least about 50° C. (e.g., at about 65° C.). The temperature may be insufficient to boil the liquid in the presence of the retention member during heating. The temperature may be 100° C. or less (e.g., less than 100° C., about 97° C. or less). The temperature may be maintained for less than about 10 minutes (e.g., for less than about 5 minutes, for less than about 3 minutes).

The releasing may be performed without centrifugation of the retention member.

In certain embodiments, PCR inhibitors are rapidly removed from clinical samples to create a PCR-ready sample. The method may comprise the preparation of a polynucleotide-containing sample that is substantially free of inhibitors. The samples may be prepared from, e.g., crude lysates resulting from thermal, chemical, ultrasonic, mechanical, electrostatic, and other lysing techniques. The samples may be prepared without centrifugation. The samples may be prepared using microfluidic devices or on a larger scale.

Another aspect of the invention relates to a retention member, e.g., a plurality of particles such as beads, comprising bound PEI, or poly-lysine, e.g., poly-L-lysine, and related methods and systems. The retention member preferentially binds polynucleotides, e.g., DNA, as compared to inhibitors. The retention member may be used to prepare polynucleotides samples for further processing, such as amplification by polymerase chain reaction.

In certain embodiments, more than 90% of a polynucleotide present in a sample may be bound to the retention member, released, and recovered.

In certain embodiments, a polynucleotide may be bound to the retention member, released, and recovered, in less than about 10 minutes (e.g., less than about 7.5 minutes, less than about 5 minutes, or less than about 3 minutes).

A polynucleotide may be bound to a retention member, released, and recovered without subjecting the polynucleotide, retention member, and/or inhibitors to centrifugation.

Separating the polynucleotides and inhibitors generally excludes subjecting the polynucleotides, inhibitors, processing region, and/or retention member to sedimentation (e.g., centrifugation).

Another aspect of the invention relates to a microfluidic device including a channel, a first mass of a thermally responsive substance (TRS) disposed on a first side of the channel, a second mass of a TRS disposed on a second side of the channel opposite the first side of the channel, a gas pressure source associated with the first mass of the TRS. Actuation of the gas pressure source drives the first mass of the TRS into the second mass of the TRS and obstructs the channel.

The microfluidic device can include a second gas pressure source associated with the second mass of the TRS. Actuation of the second gas pressure source drives the second mass of TRS into the first mass of TRS.

At least one (e.g., both) of the first and second masses of TRS may be a wax.

4

Another aspect of the invention relates to a method for obstructing a channel of a microfluidic device. A mass of a TRS is heated and driven across the channel (e.g., by gas pressure) into a second mass of TRS. The second mass of TRS may also be driven (e.g., by gas pressure) toward the first mass of TRS.

Another aspect of the invention relates to an actuator for a microfluidic device. The actuator includes a channel, a chamber connected to the channel, at least one reservoir of encapsulated liquid disposed in the chamber, and a gas surrounding the reservoir within the chamber. Heating the chamber expands the reservoir of encapsulated liquid and pressurizes the gas. Typically the liquid has a boiling point of about 90° C. or less. The liquid may be a hydrocarbon having about 10 carbon atoms or fewer.

The liquid may be encapsulated by a polymer.

The actuator may include multiple reservoirs of encapsulated liquid disposed in the chamber.

The multiple reservoirs may be dispersed within a solid (e.g., a wax).

The multiple reservoirs may be disposed within a flexible enclosure (e.g., a flexible sack).

Another aspect of the invention relates to a method including pressurizing a gas within a chamber of a microfluidic to create a gas pressure sufficient to move a liquid within a channel of the microfluidic device. Pressurizing the gas typically expanding at least one reservoir of encapsulated liquid disposed within the chamber.

Expanding the at least one reservoir can include heating the chamber.

Pressurizing the gas can include expanding multiple reservoirs of encapsulated liquid.

Another aspect of the invention relates to a method for combining (e.g., mixing) first and second liquids and related devices. The device includes a mass of a temperature responsive substance (TRS) that separates first and second channels of the device. The device is configured to move a first liquid along the first channel so that a portion (e.g., a medial portion) of the first liquid is adjacent the TRS and to move a second liquid along the second channel so that a portion (e.g., a medial portion) of second liquid is adjacent the TRS. A heat source is actuated to move the TRS (e.g., by melting, dispersing, fragmenting). The medial portions of the first and second liquids typically combine without being separated by a gas interface. Typically, only a subset of the first liquid and a subset of the second liquid are combined. The liquids mix upon being moved along a mixing channel.

Another aspect of the invention relates to a lyophilized reagent particle and a method of making the particle.

In some embodiments, the lyophilized particles include multiple smaller particles each having a plurality of ligands that preferentially associate with polynucleotides as compared to PCR inhibitors. The lyophilized particles can also (or alternatively) include lysing reagents (e.g., enzymes) configured to lyse cells to release polynucleotides. The lyophilized particles can also (or alternatively) include enzymes (e.g., proteases) that degrade proteins.

Cells can be lysed by combining a solution of the cells with the lyophilized particles to reconstitute the particles. The reconstituted lysing reagents lyse the cells. The polynucleotides associate with ligands of the smaller particles. During lysis, the solution may be heated (e.g., radiatively using a lamp (e.g., a heat lamp)).

In some embodiments, lyophilized particles include reagents (e.g., primers, control plasmids, polymerase enzymes) for performing PCR.

US 10,604,788 B2

5

A method for making lyophilized particles includes forming a solution of reagents of the particle and a cryoprotectant (e.g., a sugar or poly-alcohol). The solution is deposited dropwise on a chilled hydrophobic surface (e.g., a diamond film or polytetrafluoroethylene surface), without contacting a cooling agent such as liquid nitrogen. The particles freeze and are subjected to reduced pressure (typically while still frozen) for a time sufficient to remove (e.g., sublimate) the solvent. The lyophilized particles may have a diameter of about 5 mm or less (e.g., about 2.5 mm or less, about 1.75 mm or less).

Another aspect of the invention relates to a liquid reservoir capable of holding a liquid (e.g., a solvent, a buffer, a reagent, or combination thereof). In general, the reservoir can have one or more of the following features.

The reservoir can include a wall that can be manipulated (e.g., pressed or depressed) to decrease a volume within the reservoir. For example, the reservoir can include a piercing member (e.g., a needle-like or otherwise pointed or sharp member) that ruptures another portion of the reservoir (e.g., a portion of the wall) to release liquid. The piercing member can be internal to the reservoir such that the piercing member ruptures the wall from an inner surface of the reservoir (e.g., wall) outwards.

In general, the wall resists passage of liquid or vapor therethrough. In some embodiments, the wall lacks stretchiness. The wall may be flexible. The wall may be, e.g., a metallic layer, e.g., a foil layer, a polymer, or a laminate including a combination thereof.

The wall may be formed by vacuum formation (e.g., applying a vacuum and heat to a layer of material to draw the layer against a molding surface). The molding surface may be concave such that the wall is provided with a generally convex surface.

Exemplary liquids held by the reservoir include water and aqueous solutions including one or more salts (e.g., magnesium chloride, sodium chloride, Tris buffer, or combination thereof). The reservoir can retain the liquid (e.g., without substantial evaporation thereof) for a period of time (e.g., at least 6 months or at least a year). In some embodiments, less than 10% (e.g., less than about 5%) by weight of the liquid evaporates over a year.

The piercing member may be an integral part of a wall of the reservoir. For example, the reservoir can include a wall having an internal projection, which may be in contact with liquid in the reservoir. The reservoir also includes a second wall opposite the piercing member. During actuation, the piercing member is driven through the second wall (e.g., from the inside out) to release liquid.

In some embodiments, a maximum amount of liquid retained by a reservoir is less than about 1 ml. For example, a reservoir may hold about 500 microliters or less (e.g., 300 microliters or less). Generally, a reservoir holds at least about 25 microliters (e.g., at least about 50 microliters). The reservoir can introduce within about 10% of the intended amount of liquid (e.g., 50 ± 5 μ l).

The reservoir can deliver a predetermined amount of liquid that is substantially air-free (e.g., substantially gas-free). Upon introduction of the liquid, the substantially air and/or gas free liquid produces few or no bubbles large enough to obstruct movement of the liquid within the microfluidic device. Use of a piercing member internal to the reservoir can enhance an ability of the reservoir to deliver substantially air and/or gas free liquids.

In some embodiments, the reservoir can be actuated to release liquid by pressing (e.g., by one's finger or thumb or by mechanical pressure actuation). The pressure may be

6

applied directly to a wall of the reservoir or to a plunger having a piercing member. In embodiments, minimal pressure is required to actuate the reservoir. An automated system can be used to actuate (e.g., press upon) a plurality of reservoirs simultaneously or in sequence.

In some embodiments, the reservoir does not include a piercing member. Instead, internal pressure generated within the reservoir ruptures a wall of the reservoir allowing liquid to enter the microfluidic device.

Upon actuating a reservoir to introduce liquid into the microfluidic device, liquid generally does not withdraw back into the reservoir. For example, upon actuation, the volume of the reservoir may decrease to some minimum but generally does not increase so as to withdraw liquid back into the reservoir. For example, the reservoir may stay collapsed upon actuation. In such embodiments, the flexible wall may be flexible but lack hysteresis or stretchiness. Alternatively or in combination, the reservoir may draw in air from a vent without withdrawing any of the liquid.

Actuation of the reservoir may include driving a piercing member through a wall of the reservoir.

The reservoir preserves the reactivity and composition of reagents therein (e.g., the chemicals within the reservoir may exhibit little or no change in reactivity over 6 months or a year).

The flexible wall of the reservoir can limit or prevent leaching of chemicals therethrough. The reservoir can be assembled independently of a microfluidic device and then secured to the microfluidic device.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of a microfluidic device.

FIG. 2 is a cross-sectional view of a processing region for retaining polynucleotides and/or separating polynucleotides from inhibitors.

FIG. 3 is a cross-sectional view of an actuator.

FIG. 4 is a perspective view of a microfluidic device.

FIG. 5 is a side cross-sectional view of the microfluidic device of FIG. 4.

FIGS. 6A and 6B, taken together, illustrate a perspective view of a microfluidic network of the microfluidic device of FIG. 4.

FIG. 7 illustrates an array of heat sources for operating components of the microfluidic device of FIG. 4.

FIGS. 8 and 9 illustrate a valve in the open and closed states respectively.

FIG. 10A-10D illustrate a mixing gate of the microfluidic network of FIGS. 6A and 6B and adjacent regions of the network.

FIGS. 11A-11C illustrate a reservoir with actuation mechanism.

FIGS. 12A-12C illustrate a reservoir with actuation mechanism.

FIG. 13 illustrates a reservoir with actuation mechanism.

FIGS. 14A-14B illustrate a reservoir with actuation mechanism.

FIGS. 15A-15B illustrate a reservoir with actuation mechanism.

FIG. 16 illustrates a reservoir with actuation mechanism.

FIG. 17 illustrates a reservoir with actuation mechanism.

FIG. 18 illustrates a device for separating polynucleotides and inhibitors.

FIG. 19 illustrates the device of FIG. 18 and a device for operation thereof.

FIG. 20 illustrates a microfluidic device.

US 10,604,788 B2

7

FIG. 21 is a cross-section of the microfluidic device of FIG. 20 taken along 5.

FIG. 22 illustrates the retention of herring sperm DNA.

FIG. 23 illustrates the retention and release of DNA from group B streptococci;

FIG. 24 illustrates the PCR response of a sample from which inhibitors had been removed and of a sample from which inhibitors had not been removed.

FIG. 25 illustrates the PCR response of a sample prepared in accord with the invention and a sample prepared using a commercial DNA extraction method.

FIG. 26A illustrates a flow chart showing steps performed during a method for separating polynucleotides and inhibitors.

FIG. 26B illustrates DNA from samples subjected to the method of FIG. 26A.

FIGS. 27A and 27B show, respectively, two embodiments of a reservoir with a piercing member.

DETAILED DESCRIPTION OF THE INVENTION

Analysis of biological samples often includes determining whether one or more polynucleotides (e.g., a DNA, RNA, mRNA, or rRNA) is present in the sample. For example, one may analyze a sample to determine whether a polynucleotide indicative of the presence of a particular pathogen is present. Typically, biological samples are complex mixtures. For example, a sample may be provided as a blood sample, a tissue sample (e.g., a swab of, for example, nasal, buccal, anal, or vaginal tissue), a biopsy aspirate, a lysate, as fungi, or as bacteria. Polynucleotides to be determined may be contained within particles (e.g., cells (e.g., white blood cells and/or red blood cells), tissue fragments, bacteria (e.g., gram positive bacteria and/or gram negative bacteria), fungi, spores). One or more liquids (e.g., water, a buffer, blood, blood plasma, saliva, urine, spinal fluid, or organic solvent) is typically part of the sample and/or is added to the sample during a processing step.

Methods for analyzing biological samples include providing a biological sample (e.g., a swab), releasing polynucleotides from particles (e.g., bacteria) of the sample, amplifying one or more of the released polynucleotides (e.g., by polymerase chain reaction (PCR)), and determining the presence (or absence) of the amplified polynucleotide(s) (e.g., by fluorescence detection). Biological samples, however, typically include inhibitors (e.g., mucosal compounds, hemoglobin, faecal compounds, and DNA binding proteins) that can inhibit determining the presence of polynucleotides in the sample. For example, such inhibitors can reduce the amplification efficiency of polynucleotides by PCR and other enzymatic techniques for determining the presence of polynucleotides. If the concentration of inhibitors is not reduced relative to the polynucleotides to be determined, the analysis can produce false negative results.

We describe methods and related systems for processing biological samples (e.g., samples having one or more polynucleotides to be determined). Typically, the methods and systems reduce the concentration of inhibitors relative to the concentration of polynucleotides to be determined.

Referring to FIG. 1, a microfluidic device 200 includes first, second, and third layers 205, 207, and 209 that define a microfluidic network 201 having various components configured to process a sample including one or more polynucleotides to be determined. Device 200 typically processes the sample by increasing the concentration of a polynucleotide to be determined and/or by reducing the

8

concentration of inhibitors relative to the concentration of polynucleotide to be determined.

We now discuss the arrangement of components of network 201.

Network 201 includes an inlet 202 by which sample material can be introduced to the network and an output 236 by which a processed sample can be removed (e.g., expelled by or extracted from) network 201. A channel 204 extends between inlet 202 and a junction 255. A valve 206 is positioned along channel 204. A reservoir channel 240 extends between junction 255 and an actuator 244. Gates 242 and 246 are positioned along channel 240. A channel 257 extends between junction 255 and a junction 259. A valve 208 is positioned along channel 257. A reservoir channel 246 extends between junction 259 and an actuator 248. Gates 250 and 252 are positioned along channel 246. A channel 261 extends between junction 259 and a junction 263. A valve 210 and a hydrophobic vent 212 are positioned along channel 261. A channel 256 extends between junction 263 and an actuator 254. A gate 258 is positioned along channel 256.

A channel 214 extends between junction 263 and a processing chamber 220, which has an inlet 265 and an outlet 267. A channel 228 extends between processing chamber outlet 267 and a waste reservoir 232. A valve 234 is positioned along channel 228. A channel 230 extends between processing chamber outlet 267 and output 236.

We turn now to particular components of network 201.

Referring also to FIG. 2, processing chamber 220 includes a plurality of particles (e.g., beads, microspheres) 218 configured to retain polynucleotides of the sample under a first set of conditions (e.g., a first temperature and/or first pH) and to release the polynucleotides under a second set of conditions (e.g., a second, higher temperature and/or a second, more basic pH). Typically, the polynucleotides are retained preferentially as compared to inhibitors that may be present in the sample. Particles 218 are configured as a retention member 216 (e.g., a column) through which sample material (e.g., polynucleotides) must pass when moving between the inlet 265 and outlet 267 of processing region 220.

A filter 219 prevents particles 218 from passing downstream of processing region 220. A channel 287 connects filter 219 with outlet 267. Filter 219 has a surface area within processing region 220 that is larger than the cross-sectional area of inlet 265. For example, in some embodiments, the ratio of the surface area of filter 219 within processing region 220 to the cross-sectional area of inlet 265 (which cross-sectional area is typically about the same as the cross-sectional area of channel 214) is at least about 5 (e.g., at least about 10, at least about 20, at least about 30). In some embodiments, the surface area of filter 219 within processing region 220 is at least about 1 mm² (e.g., at least about 2 mm², at least about 3 mm²). In some embodiments, the cross-sectional area of inlet 265 and/or channel 214 is about 0.25 mm² or less (e.g., about 0.2 mm² or less, about 0.15 mm² or less, about 0.1 mm² or less). The larger surface area presented by filter 219 to material flowing through processing region 220 helps prevent clogging of the processing region while avoiding significant increases in the void volume (discussed below) of the processing region.

Particles 218 are modified with at least one ligand that retains polynucleotides (e.g., preferentially as compared to inhibitors). Typically, the ligands retain polynucleotides from liquids having a pH about 9.5 or less (e.g., about 9.0 or less, about 8.75 or less, about 8.5 or less). As a sample solution moves through processing region 220, polynucle-

otides are retained while the liquid and other solution components (e.g., inhibitors) are less retained (e.g., not retained) and exit the processing region. In general, the ligands release polynucleotides when the pH is about 10 or greater (e.g., about 10.5 or greater, about 11.0 or greater, about 11.4 or greater). Consequently, polynucleotides can be released from the ligand modified particles into the surrounding liquid.

Exemplary ligands include, for example, polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine) and PEI. Other ligands include, for example, intercalators, poly-intercalators, minor groove binders polyamines (e.g., spermidine), homopolymers and copolymers comprising a plurality of amino acids, and combinations thereof. In some embodiments, the ligands have an average molecular weight of at least about 5000 Da (e.g., at least about 7500 Da, of at least about 15000 Da). In some embodiments, the ligands have an average molecular weight of about 50000 Da or less (e.g., about 35000, or less, about 27500 Da or less). In some embodiments, the ligand is a poly-lysine ligand attached to the particle surface by an amide bond.

In certain embodiments, the ligands are resistant to enzymatic degradation, such as degradation by protease enzymes (e.g., mixtures of endo- and exo-proteases such as pronase) that cleave peptide bonds. Exemplary protease resistant ligands include, for example, poly-D-lysine and other ligands that are enantiomers of ligands susceptible to enzymatic attack.

Particles **218** are typically formed of a material to which the ligands can be associated. Exemplary materials from which particles **218** can be formed include polymeric materials that can be modified to attach a ligand. Typical polymeric materials provide or can be modified to provide carboxylic groups and/or amino groups available to attach ligands. Exemplary polymeric materials include, for example, polystyrene, latex polymers (e.g., polycarboxylate coated latex), polyacrylamide, polyethylene oxide, and derivatives thereof. Polymeric materials that can be used to form particles **218** are described in U.S. Pat. No. 6,235,313 to Mathiowitz et al., which patent is incorporated herein by reference. Other materials include glass, silica, agarose, and amino-propyl-tri-ethoxy-silane (APES) modified materials.

Exemplary particles that can be modified with suitable ligands include carboxylate particles (e.g., carboxylate modified magnetic beads (Sera-Mag Magnetic Carboxylate modified beads, Part #3008050250, Seradyn) and Polybead carboxylate modified microspheres available from Polyscience, catalog no. 09850). In some embodiments, the ligands include poly-D-lysine and the beads comprise a polymer (e.g., polycarboxylate coated latex). In other embodiments, the ligands include PEI.

In general, the ratio of mass of particles to the mass of polynucleotides retained by the particles is no more than about 25 or more (e.g., no more than about 20, no more than about 10). For example, in some embodiments, about 1 gram of particles retains about 100 milligrams of polynucleotides.

Typically, the total volume of processing region **220** (including particles **218**) between inlet **265** and filter **219** is about 15 microliters or less (e.g., about 10 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. In some embodiments, particles **218** occupy at least about 10 percent (e.g., at least about 15 percent) of the total volume of processing region **220**. In some embodiments,

particles **218** occupy about 75 percent or less (e.g., about 50 percent or less, about 35 percent or less) of the total volume of processing chamber **220**.

In some embodiments, the volume of processing region **220** that is free to be occupied by liquid (e.g., the void volume of processing region **220** including interstices between particles **218**) is about equal to the total volume minus the volume occupied by the particles. Typically, the void volume of processing region **220** is about 10 microliters or less (e.g., about 7.5 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In some embodiments, the void volume is about 50 nanoliters or more (e.g., about 100 nanoliters or more, about 250 nanoliters or more). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. For example, in an exemplary embodiment, the total volume of the processing region is about 2.3 microliters, the volume occupied by particles is about 0.3 microliters, and the volume free to be occupied by liquid (void volume) is about 2 microliters.

Particles **218** typically have an average diameter of about 20 microns or less (e.g., about 15 microns or less, about 10 microns or less). In some embodiments, particles **218** have an average diameter of at least about 4 microns at least about 6 microns, at least about 8 microns).

In some embodiments, a volume of channel **287** between filter **219** and outlet **267** is substantially smaller than the void volume of processing region **220**. For example, in some embodiments, the volume of channel **287** between filter **219** and outlet **267** is about 35% or less (e.g., about 25% or less, about 20% or less) of the void volume. In an exemplary embodiment, the volume of channel **287** between filter **219** and outlet **267** is about 500 nanoliters.

The particle density is typically at least about 10^8 particles per milliliter (e.g., about 10^9 particles per milliliter). For example, a processing region with a total volume of about 1 microliter may include about 103 beads.

Filter **219** typically has pores with a width smaller than the diameter of particles **218**. In an exemplary embodiment, filter **219** has pores having an average width of about 8 microns and particles **218** have an average diameter of about 10 microns.

In some embodiments, at least some (e.g., all) of the particles are magnetic. In alternative embodiments, few (e.g., none) of the particles are magnetic.

In some embodiments, at least some (e.g., all) the particles are solid. In some embodiments, at least some (e.g., all) the particles are porous (e.g., the particles may have channels extending at least partially within them).

We continue discussing components of network **201**.

Channels of microfluidic network **201** typically have at least one sub-millimeter cross-sectional dimension. For example, channels of network **201** may have a width and/or a depth of about 1 mm or less (e.g., about 750 microns or less, about 500 microns, or less, about 250 microns or less).

A valve is a component that has a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Upon actuation, the valve transitions to a closed state that prevents material from passing along the channel from one side of the valve to the other. For example, valve **206** includes a mass **251** of a thermally responsive substance (TRS) that is relatively immobile at a first temperature and more mobile at a second temperature. A chamber **253** is in gaseous communication with mass **251**. Upon heating gas (e.g., air) in chamber **253** and heating mass **251** of TRS to

US 10,604,788 B2

11

the second temperature, gas pressure within chamber **253** moves mass **251** into channel **204** obstructing material from passing therealong. Other valves of network **201** have the same structure and operate in the same fashion as valve **206**.

A mass of TRS can be an essentially solid mass or an agglomeration of smaller particles that cooperate to obstruct the passage. Examples of TRS's include a eutectic alloy (e.g., a solder), wax (e.g., an olefin), polymers, plastics, and combinations thereof. The first and second temperatures are insufficiently high to damage materials, such as polymer layers of device **200**. Generally, the second temperature is less than about 90° C. and the first temperature is less than the second temperature (e.g., about 70° C. or less).

A gate is a component that has a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Upon actuation, the gate transitions to an open state in which material is permitted to pass from one side of the gate (e.g., upstream of the gate) to the other side of the gate (e.g., downstream of the gate). For example, gate **242** includes a mass **271** of TRS positioned to obstruct passage of material between junction **255** and channel **240**. Upon heating mass **271** to the second temperature, the mass changes state (e.g., by melting, by dispersing, by fragmenting, and/or dissolving) to permit passage of material between junction **255** and channel **240**.

The portion of channel **240** between gates **242** and **246** forms a fluid reservoir **279** configured to hold a liquid (e.g., water, an organic liquid, or combination thereof). During storage, gates **242** and **246** limit (e.g., prevent) evaporation of liquid within the fluid reservoir. During operation of device **200**, the liquid of reservoir **279** is typically used as a wash liquid to remove inhibitors from processing region **220** while leaving polynucleotides associated with particles **218**. Typically, the wash liquid is a solution having one or more additional components (e.g., a buffer, chelator, surfactant, a detergent, a base, an acid, or a combination thereof). Exemplary solutions include, for example, a solution of 10-50 mM Tris at pH 8.0, 0.5-2 mM EDTA, and 0.5%-2% SDS, a solution of 10-50 mM Tris at pH 8.0, 0.5 to 2 mM EDTA, and 0.5%-2% Triton X-100.

The portion of channel **246** between gates **250** and **252** form a fluid reservoir **281** configured like reservoir **279** to hold a liquid (e.g., a solution) with limited or no evaporation. During operation of device **200**, the liquid of reservoir **281** is typically used as a release liquid into which polynucleotides that had been retained by particles **218** are released. An exemplary release liquid is an hydroxide solution (e.g., a NaOH solution) having a concentration of, for example, between about 2 mM hydroxide (e.g., about 2 mM NaOH) and about 500 mM hydroxide (e.g., about 500 mM NaOH). In some embodiments, liquid in reservoir **281** is an hydroxide solution having a concentration of about 25 mM or less (e.g., an hydroxide concentration of about 15 mM).

Reservoirs **279**, **281** typically hold at least about 0.375 microliters of liquid (e.g., at least about 0.750 microliters, at least about 1.25 microliters, at least about 2.5 microliters). In some embodiments, reservoirs **279**, **281** hold about 7.5 microliters or less of liquid (e.g., about 5 microliters or less, about 4 microliters or less, about 3 microliters or less).

An actuator is a component that provides a gas pressure that can move material (e.g., sample material and/or reagent material) between one location of network **201** and another location. For example, referring to FIG. 3, actuator **244** includes a chamber **272** having a mass **273** of thermally expansive material (TEM) therein. When heated, the TEM expands decreasing the free volume within chamber **272** and

12

pressurizing the gas (e.g., air) surrounding mass **273** within chamber **272**. Typically, gates **246** and **242** are actuated with actuator **244**. Consequently, the pressurized gas drives liquid in fluid reservoir **279** towards junction **255**. In some embodiments, actuator **244** can generate a pressure differential of more than about 3 psi (e.g., at least about 4 psi, at least about 5 psi) between the actuator and junction **255**.

The TEM includes a plurality of sealed liquid reservoirs (e.g., spheres) **275** dispersed within a carrier **277**. Typically, the liquid is a high vapor pressure liquid (e.g., isobutane and/or isopentane) sealed within a casing (e.g., a polymeric casing formed of monomers such as vinylidene chloride, acrylonitrile and methylmethacrylate). Carrier **277** has properties (e.g., flexibility and/or an ability to soften (e.g., melt) at higher temperatures) that permit expansion of the reservoirs **275** without allowing the reservoirs to pass along channel **240**. In some embodiments, carrier **277** is a wax (e.g., an olefin) or a polymer with a suitable glass transition temperature. Typically, the reservoirs make up at least about 25 weight percent (e.g., at least about 35 weight percent, at least about 50 weight percent) of the TEM. In some embodiments, the reservoirs make up about 75 weight percent or less (e.g., about 65 weight percent or less, about 50 weight percent or less) of the TEM. Suitable sealed liquid reservoirs can be obtained from Expancel (Akzo Nobel).

When the TEM is heated (e.g., to a temperature of at least about 50° C. (e.g., to at least about 75° C., at least about 90° C.)), the liquid vaporizes and increases the volume of each sealed reservoir and of mass **273**. Carrier **277** softens allowing mass **273** to expand. Typically, the TEM is heated to a temperature of less than about 150° C. (e.g., about 125° C. or less, about 110° C. or less, about 100° C. or less) during actuation. In some embodiments, the volume of the TEM expands by at least about 5 times (e.g., at least about 10 times, at least about 20 times, at least about 30 times).

A hydrophobic vent (e.g., vent **212**) is a structure that permits gas to exit a channel while limiting (e.g., preventing) liquid from exiting the channel. Typically, hydrophobic vents include a layer of porous hydrophobic material (e.g., a porous filter such as a porous hydrophobic membrane from Osmonics) that defines a wall of the channel. As discussed below, hydrophobic vents can be used to position a microdroplet of sample at a desired location within network **201**.

The hydrophobic vents of the present invention are preferably constructed so that the amount of air that escapes through them is maximized while minimizing the volume of the channel below the vent surface. Accordingly, it is preferable that the vent is constructed so as to have a hydrophobic membrane of large surface area and a shallow cross section of the microchannel below the vent surface.

Hydrophobic vents typically have a length of at least about 2.5 mm (e.g., at least about 5 mm, at least about 7.5 mm) along a channel. The length of the hydrophobic vent is typically at least about 5 times (e.g., at least about 10 times, at least about 20 times) larger than a depth of the channel within the hydrophobic vent. For example, in some embodiments, the channel depth within the hydrophobic vent is about 300 microns or less (e.g., about 250 microns or less, about 200 microns or less, about 150 microns or less).

The depth of the channel within the hydrophobic vent is typically about 75% or less about 65% or less, about 60% or less) of than the depth of the channel upstream and downstream of the hydrophobic vent. For example, in some embodiments the channel depth within the hydrophobic vent is about 150 microns and the channel depth upstream and downstream of the hydrophobic vent is about 250 microns.

US 10,604,788 B2

13

A width of the channel within the hydrophobic vent is typically at least about 25% wider (e.g., at least about 50% wider) than a width of the channel upstream from the vent and downstream from the vent. For example, in an exemplary embodiment, the width of the channel within the hydrophobic vent is about 400 microns and the width of the channel upstream and downstream from the vent is about 250 microns.

Microfluidic device **200** can be fabricated as desired. Typically, layers **205**, **207**, and **209** are formed of a polymeric material. Components of network **201** are typically formed by molding (e.g., by injection molding) layers **207**, **209**. Layer **205** is typically a flexible polymeric material (e.g., a laminate) that is secured (e.g., adhesively and/or thermally) to layer **207** to seal components of network **201**. Layers **207** and **209** may be secured to one another using adhesive.

In use, device **200** is typically thermally associated with an array of heat sources configured to operate the components (e.g., valves, gates, actuators, and processing region **220**) of the device. In some embodiments, the heat sources are operated by an operating system, which operates the device during use. The operating system includes a processor (e.g., a computer) configured to actuate the heat sources according to a desired protocol. Processors configured to operate microfluidic devices are described in U.S. application Ser. No. 09/819,105, filed Mar. 28, 2001, which application is incorporated herein by reference. In other embodiments, the heat sources are integral with the device itself.

Device **200** may be operated as follows. Valves of network **201** are configured in the open state. Gates of network **201** are configured in the closed state. A fluidic sample comprising polynucleotides is introduced to network **201** via inlet **202**. For example, sample can be introduced with a syringe having a Luer fitting. The syringe provides pressure to initially move the sample within network **201**. Sample passes along channels **204**, **257**, **261**, and **214** to inlet **265** of processing region **220**. The sample passes through processing region **220**, exits via outlet **267**, and passes along channel **228** to waste chamber **232**. When the trailing edge (e.g., the upstream liquid-gas interface) of the sample reaches hydrophobic vent **212**, pressure provided by the introduction device (e.g., the syringe) is released from network **201** stopping further motion of the sample.

Typically, the amount of sample introduced is about 500 microliters or less (e.g., about 250 microliters or less, about 100 microliters or less, about 50 microliters or less, about 25 microliters or less, about 10 microliters or less). In some embodiments, the amount of sample is about 2 microliters or less (e.g., of about 0.5 microliters or less).

Polynucleotides entering processing region **220** pass through interstices between the particles **218**. Polynucleotides of the sample contact retention member **216** and are preferentially retained as compared to liquid of the sample and certain other sample components (e.g., inhibitors). Typically, retention member **220** retains at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **220**. Liquid of the sample and inhibitors present in the sample exit the processing region **220** via outlet **267** and enter waste chamber **232**. Processing region **220** is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during introduction of the sample.

Processing continues by washing retention member **216** with liquid of reservoir **279** to separate remaining inhibitors from polynucleotides retained by retention member **216**. To

14

wash retention member **216**, valve **206** is closed and gates **242**, **246** of first reservoir **240** are opened. Actuator **244** is actuated and moves wash liquid within reservoir **279** along channels **257**, **261**, and **214**, through processing region **220**, and into waste reservoir **232**. The wash liquid moves sample that may have remained within channels **204**, **257**, **261**, and **214** through the processing region and into waste chamber **232**. Once the trailing edge of the wash liquid reaches vent **212**, the gas pressure generated by actuator **244** is vented and further motion of the liquid is stopped.

The volume of wash liquid moved by actuator **244** through processing region **220** is typically at least about 2 times the void volume of processing region **220** (e.g., at least about 3 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less). Processing region is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during washing. Exemplary wash fluids include liquids discussed with respect to reservoirs **279** and **281**.

Processing continues by releasing polynucleotides from retention member **216**. Typically, wash liquid from reservoir **279** is replaced with release liquid (e.g., an hydroxide solution) from reservoir **281** before releasing the polynucleotides. Valve **208** is closed and gates **250**, **252** are opened. Actuator **248** is actuated thereby moving release liquid within reservoir **281** along channels **261**, **214** and into processing region **220** and in contact with retention member **216**. When the trailing edge of release liquid from reservoir **281** reaches hydrophobic vent **212**, pressure generated by actuator **248** is vented stopping the further motion of the liquid. The volume of liquid moved by actuator **248** through processing region **220** is typically at least about equal to the void volume of the processing region **220** (e.g., at least about 2 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less).

Once retention member **216** with retained polynucleotides has been contacted with liquid from reservoir **281**, a releasing step is typically performed. Typically, the releasing step includes heating release liquid present within processing region **216**. Generally, the liquid is heated to a temperature insufficient to boil liquid in the presence of the retention member. In some embodiments, the temperature is 100° C. or less (e.g., less than 100° C., about 97° C. or less). In some embodiments, the temperature is about 65° C. or more (e.g., about 75° C. or more, about 80° C. or more, about 90° C. or more). In some embodiments, the temperature maintained for about 1 minute or more (e.g., about 2 minutes or more, about 5 minutes or more, about 10 minutes or more). In some embodiments, the temperature is maintained for about 30 minutes (e.g., about 15 minutes or less, about 10 minutes or less, about 5 minutes or less). In an exemplary embodiment, processing region **220** is heated to between about 65 and 90° C. (e.g., to about 70° C.) for between about 1 and 7 minutes (e.g., for about 2 minutes).

The polynucleotides are released into the liquid present in the processing region **220** (e.g., the polynucleotides are typically released into an amount of release liquid having a volume about the same as the void volume of the processing region **220**). Typically, the polynucleotides are released into about 10 microliters or less (e.g., about 5 microliters or less, about 2.5 microliters or less) of liquid.

In certain embodiments, the ratio of the volume of original sample moved through the processing region **220** to the volume of liquid into which the polynucleotides are released is at least about 10 (e.g., at least about 50, at least about 100, at least about 250, at least about 500, at least about 1000). In some embodiments, polynucleotides from a sample hav-

ing a volume of about 2 ml can be retained within the processing region, and released into about 4 microliters or less (e.g., about 3 microliters or less, about 2 microliters or less, about 1 microliter or less) of liquid.

The liquid into which the polynucleotides are released typically includes at least about 50% (e.g., at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region 220. The concentration of polynucleotides present in the release liquid may be higher than in the original sample because the volume of release liquid is typically less than the volume of the original liquid sample moved through the processing region. For example the concentration of polynucleotides in the release liquid may be at least about 10 times greater (e.g., at least about 25 times greater, at least about 100 times greater) than the concentration of polynucleotides in the sample introduced to device 200. The concentration of inhibitors present in the liquid into which the polynucleotides are released is generally less than concentration of inhibitors in the original fluidic sample by an amount sufficient to increase the amplification efficiency for the polynucleotides.

The time interval between introducing the polynucleotide containing sample to processing region 220 and releasing the polynucleotides into the release liquid is typically about 15 minutes or less (e.g., about 10 minutes or less, about 5 minutes or less).

Liquid including the released polynucleotides may be removed from the processing region 220 as follows. Valves 210 and 234 are closed. Gates 238 and 258 are opened. Actuator 254 is actuated to generate pressure that moves liquid and polynucleotides from processing region 220, into channel 230, and toward outlet 236. The liquid with polynucleotides can be removed using, for example, a syringe or automated sampling device. Depending upon the liquid in contact with retention member 216 during polynucleotide release, the solution with released polynucleotide may be neutralized with an amount of buffer (e.g., an equal volume of 25-50 mM Tris-HCl buffer pH 8.0).

While releasing the polynucleotides has been described as including a heating step, the polynucleotides may be released without heating. For example, in some embodiments, the liquid of reservoir 281 has an ionic strength, pH, surfactant concentration, composition, or combination thereof that releases the polynucleotides from the retention member.

While the polynucleotides have been described as being released into a single volume of liquid present within processing region 220, other configurations can be used. For example, polynucleotides may be released with the concomitant (stepwise or continuous) introduction of fluid into and/or through processing region 220. In such embodiments, the polynucleotides may be released into liquid having a volume of about 10 times or less (e.g., about 7.5 times or less, about 5 times or less, about 2.5 times or less, about 2 times or less) than the void volume of the processing region 220.

While reservoirs 279, 281 have been described as holding liquids between first and second gates, other configurations can be used. For example, liquid for each reservoir may be held within a pouch (e.g., a blister pack) isolated from network 201 by a generally impermeable membrane. The pouch is configured so that a user can rupture the membrane driving liquid into reservoirs 279, 281 where actuators 244, 248 can move the liquid during use.

While processing regions have been described as having microliter scale dimensions, other dimensions can be used.

For example, processing regions with surfaces (e.g., particles) configured to preferentially retain polynucleotides as opposed to inhibitors may have large volumes (e.g., many tens of microliters or more, at least about 1 milliliter or more). In some embodiments, the processing region has a bench-top scale.

While processing region 220 has been described as having a retention member formed of multiple surface-modified particles, other configurations can be used. For example, in some embodiments, processing region 220 includes a retention member configured as a porous member (e.g., a filter, a porous membrane, or a gel matrix) having multiple openings (e.g., pores and/or channels) through which polynucleotides pass. Surfaces of the porous member are modified to preferentially retain polynucleotides. Filter membranes available from, for example, Osmonics, are formed of polymers that may be surface-modified and used to retain polynucleotides within processing region 220. In some embodiments, processing region 220 includes a retention member configured as a plurality of surfaces (e.g., walls or baffles) through which a sample passes. The walls or baffles are modified to preferentially retain polynucleotides.

While processing region 220 has been described as a component of a microfluidic network, other configurations can be used. For example, in some embodiments, the retention member can be removed from a processing region for processing elsewhere. For example, the retention member may be contacted with a mixture comprising polynucleotides and inhibitors in one location and then moved to another location at which the polynucleotides are removed from the retention member.

While reservoirs 275 have been shown as dispersed within a carrier, other configurations may be used. For example, reservoirs 275 can be encased within a flexible enclosure (e.g., a membrane, for example, an enclosure such as a sack). In some embodiments, reservoirs are loose within chamber 272. In such embodiments, actuator 244 may include a porous member having pores too small to permit passage of reservoirs 275 but large enough to permit gas to exit chamber 272.

Microfluidic devices with various components are described in U.S. provisional application No. 60/553,553 filed Mar. 17, 2004 by Parunak et al., which application is incorporated herein by reference.

While microfluidic device 300 has been described as configured to receive polynucleotides already released from cells, microfluidic devices can be configured to release polynucleotides from cells (e.g., by lysing the cells). For example, referring to FIGS. 4, 5, 6A, and 6B, a microfluidic device 300 includes a sample lysing chamber 302 in which cells are lysed to release polynucleotides therein. Microfluidic device 300 further includes substrate layers L1-L3, a microfluidic network 304 (only portions of which are seen in FIG. 4), and liquid reagent reservoirs R1-R4. Liquid reagent reservoirs R1-R4 hold liquid reagents (e.g., for processing sample material) and are connected to network 304 by reagent ports RP1-RP4.

Network 304 is substantially defined between layers L2 and L3 but extends in part between all three layers L1-L3. Microfluidic network 304 includes multiple components including channels Ci, valves Vi, double valves Vi, gates Gi, mixing gates MG_i, vents Hi, gas actuators (e.g., pumps) Pi, a first processing region B1, a second processing region B2, detection zones Di, air vents AV_i, and waste zones Wi.

Components of network 304 are typically thermally actuated. As seen in FIG. 7, a heat source network 312 includes heat sources (e.g., resistive heat sources) having locations

that correspond to components of microfluidic network **304**. For example, the locations of heat sources HP_i correspond to the locations of actuators P_i , the locations of heat sources HGi correspond to locations of gates Gi and mixing gates MGi , the locations of heat sources HVi correspond to the locations of valves Vi and double valves $V'i$, and the locations of heat sources HDi correspond to the locations of processing chambers Di of network **304**. In use, the components of device **300** are disposed in thermal contact with corresponding heat sources of network **312**, which is typically operated using a processor as described above for device **200**. Heat source network **312** can be integral with or separate from device **300** as described for device **200**.

We next discuss components of microfluidic device **300**.

Air vents AV_i are components that allow gas (e.g., air) displaced by the movement of liquids within network **304** to be vented so that pressure buildup does not inhibit desired movement of the liquids. For example, air vent AV_2 permits liquid to move along channel $C14$ and into channel $C16$ by venting gas downstream of the liquid through vent AV_2 .

Valves Vi are components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). The valves Vi can have the same structure as valves of microfluidic device **200**.

As seen in FIGS. **8** and **9**, double valves $V'i$ are also components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Taking double valve $V'11$ of FIGS. **8** and **9** as an example, double valves Vi include first and second masses **314**, **316** of a TRS (e.g., a eutectic alloy or wax) spaced apart from one another on either side of a channel (e.g., channel $C14$). Typically, the TRS masses **314**, **316** are offset from one another (e.g., by a distance of about 50% of a width of the TRS masses or less). Material moving through the open valve passes between the first and second TRS masses **314**, **316**. Each TRS mass **314**, **316** is associated with a respective chamber **318**, **320**, which typically includes a gas (e.g., air).

The TRS masses **314**, **316** and chambers **318**, **320** of double valve $V'i$ are in thermal contact with a corresponding heat source $HV'11$ of heat source network **312**. Actuating heat source $HV'11$ causes TRS masses **314**, **316** to transition to a more mobile second state (e.g., a partially melted state) and increases the pressure of gas within chambers **318**, **320**. The gas pressure drives TRS masses **314**, **316** across channel $C11$ and closes valve $HV'11$ (FIG. **9**). Typically, masses **314**, **316** at least partially combine to form a mass **322** that obstructs channel $C11$.

Returning to FIGS. **6A**, **6B**, gates Gi are components that have a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Gates Gi can have the same structure as described for gates of device **200**.

As seen in **10A-10D**, mixing gates MGi are components that allow two volumes of liquid to be combined (e.g., mixed) within network **304**. Mixing gates MGi are discussed further below.

Actuators P_i are components that provide a gas pressure to move material (e.g., sample material and/or reagent material) between one location of network **304** and another location. Actuators P_i can be the same as actuators of device **200**. For example, each actuator P_i includes a chamber with a mass **273** of TEM that can be heated to pressurize gas within the chamber. Each actuator P_i includes a correspond-

ing gate Gi (e.g., gate G_2 of actuator P_1) that prevents liquid from entering the chamber of the actuator. The gate is typically actuated (e.g., opened) to allow pressure created in the chamber of the actuator to enter the microfluidic network.

Waste chambers Wi are components that can receive waste (e.g., overflow) liquid resulting from the manipulation (e.g., movement and/or mixing) of liquids within network **304**. Typically, each waste chamber Wi has an associated air vent that allows gas displaced by liquid entering the chamber to be vented.

First processing region **B1** is a component that allows polynucleotides to be concentrated and/or separated from inhibitors of a sample. Processing region **B1** can be configured and operated as processing region **220** of device **200**. In some embodiments, first processing region **B1** includes a retention member (e.g., multiple particles (e.g., microspheres or beads), a porous member, multiple walls) having at least one surface modified with one or more ligands as described for processing region **220**. For example, the ligand can include one or more polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine), or polyethyleneimine. In some embodiments, particles of the retention member are disposed in lysing chamber **302** and are moved into processing region **B1** along with sample material.

Second processing region **B2** is a component that allows material (e.g., sample material) to be combined with compounds (e.g., reagents) for determining the presence of one or more polynucleotides. In some embodiments, the compounds include one or more PCR reagents (e.g., primers, control plasmids, and polymerase enzymes). Typically, the compounds are stored within processing region as one or more lyophilized particles (e.g., pellets). The particles generally have a room temperature (e.g., about 20° C.) shelf-life of at least about 6 months (e.g., at least about 12 months). Liquid entering the second processing region **132** dissolves (e.g., reconstitutes) the lyophilized compounds.

Typically, the lyophilized particle(s) of processing region **B2** have an average volume of about 5 microliters or less (e.g., about 4 microliters or less, about 3 microliters or less, about 2 microliters or less). In some embodiments, the lyophilized particle(s) of processing region **132** have an average diameter of about 4 mm or less (e.g., about 3 mm or less, about 2 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 2 microliters and an average diameter of about 1.35 mm.

Lyophilized particles for determining the presence of one or more polynucleotides typically include multiple compounds. In some embodiments, the lyophilized particles include one or more compounds used in a reaction for determining the presence of a polynucleotide and/or for increasing the concentration of the polynucleotide. For example, lyophilized particles can include one or more enzymes for amplifying the polynucleotide as by PCR.

We next discuss exemplary lyophilized particles that include exemplary reagents for the amplification of polynucleotides associated with group B streptococcus (GBS) bacteria. In some embodiments, the lyophilized particles include one or more of a cryoprotectant, one or more salts, one or more primers (e.g., GBS Primer F and/or GBS Primer R), one or more probes (e.g., GBS Probe—FAM), one or more internal control plasmids, one or more specificity controls (e.g., *Streptococcus pneumoniae* DNA as a control for PCR of GBS), one or more PCR reagents (e.g., dNTPs and/or dUTPs), one or more blocking or bulking agents (e.g., non-specific proteins (e.g., bovine serum albumin

US 10,604,788 B2

19

(BSA), RNaseA, or gelatin), and a polymerase (e.g., glycerol-free Taq Polymerase). Of course, other components (e.g., other primers and/or specificity controls) can be used for amplification of other polynucleotides.

Cryoprotectants generally help increase the stability of the lyophilized particles and help prevent damage to other compounds of the particles (e.g., by preventing denaturation of enzymes during preparation and/or storage of the particles). In some embodiments, the cryoprotectant includes one or more sugars (e.g., one or more disaccharides (e.g., trehalose, melezitose, raffinose)) and/or one or more polyalcohols (e.g., mannitol, sorbitol).

Lyophilized particles can be prepared as desired. Typically, compounds of the lyophilized particles are combined with a solvent (e.g., water) to make a solution, which is then placed (e.g., in discrete aliquots (e.g., drops) such as by pipette) onto a chilled hydrophobic surface (e.g., a diamond film or a polytetrafluorethylene surface). In general, the temperature of the surface is reduced to near the temperature of liquid nitrogen (e.g., about -150° F. or less, about -200° F. or less, about -275° F. or less), such as by use of a cooling bath of a cryogenic agent directly underneath. It is to be noted that the solution is dispensed without contacting the cryogenic agent. The solution freezes as discrete particles. The frozen particles are subjected to a vacuum while still frozen for a pressure and time sufficient to remove the solvent (e.g., by sublimation) from the pellets.

In general, the concentrations of the compounds in the solution from which the particles are made is higher than when reconstituted in the microfluidic device. Typically, the ratio of the solution concentration to the reconstituted concentration is at least about 3 (e.g., at least about 4.5). In some embodiments, the ratio is about 6.

An exemplary solution for preparing lyophilized pellets for use in the amplification of polynucleotides indicative of the presence of GBS can be made by combining a cryoprotectant (e.g., 120 mg of trehalose as dry powder), a buffer solution (e.g., 48 microliters of a solution of 1M Tris at pH 8.4, 2.5M KCl, and 200 mM MgCl₂), a first primer (e.g., 1.92 microliters of 500 micromolar GBS Primer R (Invitrogen)), a second primer (e.g., 1.92 microliters of 500 micromolar GBS Primer R (Invitrogen)), a probe (e.g., 1.92 microliters of 250 micromolar GBS Probe—FAM (IDT/Biosearch Technologies)), an control probe (e.g., 1.92 microliters of 250 micromolar Cal Orange 560 (Biosearch Technologies)), a template plasmid (e.g., 0.6 microliters of a solution of 105 copies plasmid per microliter), a specificity control (e.g., 1.2 microliters of a solution of 10 nanograms per microliter (e.g., about 5,000,000 copies per microliter) *Streptococcus pneumoniae* DNA (ATCC)), PCR reagents (e.g., 4.8 microliters of a 100 millimolar solution of dNTPs (Epicenter) and 4 microliters of a 20 millimolar solution of dUTPs (Epicenter)), a bulking agent (e.g., 24 microliters of a 50 milligram per milliliter solution of BSA (Invitrogen)), a polymerase (e.g., 60 microliters of a 5 U per microliter solution of glycerol-free Taq Polymerase (Invitrogen/Eppendorf)) and a solvent (e.g., water) to make about 400 microliters of solution. About 200 aliquots of about 2 microliters each of this solution are frozen and desolvated as described above to make 200 pellets. When reconstituted, the 200 particles make a PCR reagent solution having a total volume of about 2.4 milliliters.

As seen in FIG. 5, reagent reservoirs Ri are configured to hold liquid reagents (e.g., water, buffer solution, hydroxide solution) separated from network 304 until ready for use. Reservoirs R1 include an enclosure 329 that defines a sealed space 330 for holding liquids. Each space 330 is separated

20

from reagent port RPi and network 304 by a lower wall 333 of enclosure 329. A capping material 341 (e.g., a laminate, adhesive, or polymer layer) may overlie an upper wall of the enclosure.

A portion of enclosure 329 is formed as an actuation mechanism (e.g., a piercing member 331) oriented toward the lower wall 333 of each enclosure. When device 300 is to be used, reagent reservoirs Ri are actuated by depressing piercing member 331 to puncture wall 333. Piercing member 331 can be depressed by a user (e.g., with a thumb) or by the operating system used to operate device 300.

Wall 333 is typically formed of a material having a low vapor transmission rate (e.g., Aclar, a metallized (e.g. aluminum) laminate, a plastic, or a foil laminate) that can be ruptured or pierced. Reservoir 330 holds an amount of liquid suited for device 300. For example, the reservoir may hold up to about 200 microliters. The piercing member 331 may account for a portion (e.g., up to about 25%) of that volume.

In general, reservoirs Ri can be formed and filled as desired. For example, the upper wall of the enclosure can be sealed to the lower wall 333 (e.g., by adhesive and/or thermal sealing). Liquid can be introduced into the reservoir by, for example, an opening at the lower end of the piercing member 331. After filling, the opening can be sealed (e.g., by heat sealing through the localized application of heat or by the application of a sealing material (e.g., capping material 341)).

When wall 333 is punctured, fluid from the reservoir enters network 333. For example, as seen in FIGS. 5 and 6, liquid from reservoir R2 enters network 304 by port RP2 and travels along a channel C2. Gate G3 prevents the liquid from passing along channel C8. Excess liquid passes along channel C7 and into waste chamber W2. When the trailing edge of liquid from reservoir R2 passes hydrophobic vent H2, pressure created within the reservoir is vented stopping further motion of the liquid. Consequently, network 304 receives an aliquot of liquid reagent having a volume defined by the volume of channel C2 between a junction J1 and a junction J2. When actuator P1 is actuated, this aliquot of reagent is moved further within network 304. Reagent reservoirs R1, R3, and R4 are associated with corresponding channels, hydrophobic vents, and actuators.

In the configuration shown, reagent reservoir R1 typically holds a release liquid (e.g., a hydroxide solution as discussed above for device 200) for releasing polynucleotides retained within processing region B1. Reagent reservoir R2 typically holds a wash liquid (e.g., a buffer solution as discussed above for device 200) for removing un-retained compounds (e.g., inhibitors) from processing region B1 prior to releasing the polynucleotides. Reagent reservoir R3 typically holds a neutralization buffer (e.g., 25-50 mM Tris-HCl buffer at pH 8.0). Reagent reservoir R4 typically holds deionized water.

Lysing chamber 302 is divided into a primary lysing chamber 306 and a waste chamber 308. Material cannot pass from one of chambers 306, 308 into the other chamber without passing through at least a portion of network 304. Primary lysing chamber 306 includes a sample input port SP1 for introducing sample to chamber 306, a sample output port SP2 connecting chamber 306 to network 304, and lyophilized reagent LP that interact with sample material within chamber 306 as discussed below. Input port SP1 includes a one way valve that permits material (e.g., sample material and gas) to enter chamber 306 but limits (e.g., prevents) material from exiting chamber 308 by port SP1. Typically, port SP1 includes a fitting (e.g., a Luer fitting) configured to mate with a sample input device (e.g., a

US 10,604,788 B2

21

syringe) to form a gas-tight seal. Primary chamber 306 typically has a volume of about 5 milliliters or less (e.g., about 4 milliliters or less). Prior to use, primary chamber 306 is typically filled with a gas (e.g., air).

Waste chamber 308 includes a waste portion W6 by which liquid can enter chamber 308 from network 304 and a vent 310 by which gas displaced by liquid entering chamber 308 can exit.

Lyophilized reagent particles LP of lysing chamber 302 include one or more compounds (e.g., reagents) configured to release polynucleotides from cells (e.g., by lysing the cells). For example, particles LP can include one or more enzymes configured to reduce (e.g., denature) proteins (e.g., proteinases, proteases (e.g., pronase), trypsin, proteinase K, phage lytic enzymes (e.g., PlyGBS)), lysozymes (e.g., a modified lysozyme such as ReadyLyse), cell specific enzymes (e.g., mutanolysin for lysing group B streptococci)).

In some embodiments, particles LP alternatively or additionally include components for retaining polynucleotides as compared to inhibitors. For example, particles LP can include multiple particles 218 surface modified with ligands as discussed above for device 200. Particles LP can include enzymes that reduce polynucleotides that might compete with a polynucleotide to be determined for binding sites on the surface modified particles. For example, to reduce RNA that might compete with DNA to be determined, particles LP may include an enzyme such as an RNAase (e.g., RNAseA ISC BioExpress (Amresco)).

In an exemplary embodiment, particles LP cells include a cryoprotectant, particles modified with ligands configured to retain polynucleotides as compared to inhibitors, and one or more enzymes.

Typically, particles LP have an average volume of about 35 microliters or less (e.g., about 27.5 microliters or less, about 25 microliters or less, about 20 microliters or less). In some embodiments, the particles LP have an average diameter of about 8 mm or less (e.g., about 5 mm or less, about 4 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 20 microliters and an average diameter of about 3.5 mm.

Particles LP can be prepared as desired. Typically, the particles are prepared using a cryoprotectant and chilled hydrophobic surface as described above. For example, a solution for preparing particles LP can be prepared by combining a cryoprotectant (e.g., 6 grams of trehalose), a plurality of particles modified with ligands (e.g., about 2 milliliters of a suspension of carboxylate modified particles with poly-D-lysine ligands), a protease (e.g., 400 milligrams of pronase), an RNAase (e.g., 30 milligrams of RNAseA (activity of 120 U per milligram), an enzyme that digests peptidoglycan (e.g., ReadyLyse (e.g., 160 microliters of a 30000 U per microliter solution of ReadyLyse)), a cell specific enzyme (e.g., mutanolysin (e.g., 200 microliters of a 50 U per microliter solution of mutanolysin), and a solvent (e.g., water) to make about 20 milliliters. About 1000 aliquots of about 20 microliters each of this solution are frozen and desolvated as described above to make 1000 pellets. When reconstituted, the pellets are typically used to make a total of about 200 milliliters of solution.

In use, device 300 can be operated as follows. Valves Vi and Vi' of network 304 are configured in the open state. Gates Gi and mixing gates MG_i of network 304 are configured in the closed state. Reagent ports R1-R4 are depressed to introduce liquid reagents into network 304 as discussed above. A sample is introduced to lysing chamber 302 via port SP1 and combined with lyophilized particles LP within

22

primary lysing chamber 306. Typically, the sample includes a combination of particles (e.g., cells) and a buffer solution. For example, an exemplary sample includes about 2 parts whole blood to 3 about parts buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% SDS). Another exemplary sample includes group B streptococci and a buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% Triton X-100).

In general, the volume of sample introduced is smaller than the total volume of primary lysing chamber 306. For example, the volume of sample may be about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. A typical sample has a volume of about 3 milliliters or less (e.g., about 1.5 milliliters or less). A volume of gas (e.g., air) is generally introduced to primary chamber 306 along with the sample. Typically, the volume of gas introduced is about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. The volume of sample and gas combine to pressurize the gas already present within chamber 306. Valve 307 of port SP1 prevents gas from exiting chamber 306. Because gates G3, G4, G8, and G10 are in the closed state, the pressurized sample is prevented from entering network 304 via port SP2.

The sample dissolves particles LP in chamber 306. Reconstituted lysing reagents (e.g., ReadyLyse, mutanolysin) begin to lyse cells of the sample releasing polynucleotides. Other reagents (e.g., protease enzymes such as pronase) begin to reduce or denature inhibitors (e.g., proteins) within the sample. Polynucleotides from the sample begin to associate with (e.g., bind to) ligands of particles 218 released from particles LP. Typically, the sample within chamber 306 is heated (e.g., to at least about 50° C., to at least about 60° C.) for a period of time (e.g., for about 15 minutes or less, about 10 minutes or less, about 7 minutes or less) while lysing occurs. In some embodiments, optical energy is used at least in part to heat contents of lysing chamber 306. For example, the operating system used to operate device 300 can include a light source (e.g., a lamp primarily emitting light in the infrared) disposed in thermal and optical contact with chamber 306. Chamber 306 includes a temperature sensor TS used to monitor the temperature of the sample within chamber 306. The lamp output is increased or decreased based on the temperature determined with sensor TS.

Continuing with the operation of device 300, G2 is actuated (e.g., opened) providing a path between port SP2 of primary lysing chamber 306 and port W6 of lysing waste chamber 308. The path extends along channel C9, channel C8, through processing region B1, and channel C11. Pressure within chamber 306 drives the lysed sample material (containing lysate, polynucleotides bound to particles 218, and other sample components) along the pathway. Particles 218 (with polynucleotides) are retained within processing region B1 (e.g., by a filter) while the liquid and other components of the sample flow into waste chamber 308. After a period of time (e.g., between about 2 and about 5 minutes), the pressure in lysing chamber 306 is vented by opening gate G1 to create a second pathway between ports SP2 and W6. Double valves V1' and V8' are closed to isolate lysing chamber 302 from network 304.

Operation of device 300 continues by actuating pump P1 and opening gates G2, G3 and G9. Pump P1 drives wash liquid in channel C2 downstream of junction J1 through processing region B1 and into waste chamber W5. The wash liquid removes inhibitors and other compounds not retained by particles 218 from processing region B1. When the trailing edge of the wash liquid (e.g., the upstream interface)

US 10,604,788 B2

23

passes hydrophobic vent H14, the pressure from actuator P1 vents from network 304, stopping further motion of the liquid. Double valves V2' and V9' are closed.

Operation continues by actuating pump P2 and opening gates G6, G4 and G8 to move release liquid from reagent reservoir R1 into processing region B1 and into contact with particles 218. Air vent AV1 vents pressure ahead of the moving release liquid. Hydrophobic vent 146 vents pressure behind the trailing edge of the release liquid stopping further motion of the release liquid. Double valves V6' and V10' are closed.

Operation continues by heating processing region B1 (e.g., by heating particles 218) to release the polynucleotides from particles 218. The particles can be heated as described above for device 200. Typically, the release liquid includes about 15 mM hydroxide (e.g., NaOH solution) and the particles are heated to about 70° C. for about 2 minutes to release the polynucleotides from the particles 218.

Operation continues by actuating pump P3 and opening gates G5 and G10 to move release liquid from process region B1 downstream. Air vent AV2 vents gas pressure downstream of the release liquid allowing the liquid to move into channel C16. Hydrophobic vent H8 vents pressure from upstream of the release liquid stopping further movement. Double valve V11' and valve V14 are closed.

Referring to FIG. 10A-10D, mixing gate MG11 is used to mix a portion of release liquid including polynucleotides released from particles 218 and neutralization buffer from reagent reservoir R3. FIG. 10A shows the mixing gate MG11 region prior to depressing reagent reservoir R3 to introduce the neutralization buffer into network 304. FIG. 10B shows the mixing gate MG11 region, after the neutralization buffer has been introduced into channels C13 and C12. Double valve V13' is closed to isolate network 304 from reagent reservoir R3. Double valve V12' is closed to isolate network 304 from waste chamber W3. The neutralization buffer contacts one side of a mass 324 of TRS of gate MG11.

FIG. 10c shows the mixing gate MG11 region after release liquid has been moved into channel C16. The dimensions of microfluidic network 304 (e.g., the channel dimensions and the position of hydrophobic vent H8) are configured so that the portion of release liquid positioned between junctions J3 and J4 of channels C16 and C14 corresponds approximately to the volume of liquid in contact with particles 218 during the release step. In some embodiments, the volume of liquid positioned between junctions J3 and J4 is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions J3 and J4 is about 1.75 microliters. Typically, the liquid between junctions J3 and J4 includes at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region B1. Valve V14 is closed to isolate network 304 from air vent AV2.

Before actuating mixing gate MG11, the release liquid at junction J4 and the neutralization buffer at a junction J6 between channels C13 and C12 are separated only by mass 324 of TRS (e.g., the liquids are not spaced apart by a volume of gas). To combine the release liquid and neutralization buffer, pump P4 and gates G12, G13, and MG11 are actuated. Pump P4 drives the volume of neutralization liquid between junctions J5 and J6 and the volume of release liquid between junctions J4 and J3 into mixing channel C15 (FIG. 10D). Mass 324 of TRS typically disperses and/or melts allowing the two liquids to combine. The combined liquids

24

include a downstream interface 335 (formed by junction J3) and an upstream interface (formed by junction J5). The presence of these interfaces allows more efficient mixing (e.g., recirculation of the combined liquid) than if the interfaces were not present. As seen in FIG. 10D, mixing typically begins near the interface between the two liquids. Mixing channel C15 is typically at least about as long (e.g., at least about twice as long) as a total length of the combined liquids within the channel.

The volume of neutralization buffer combined with the release liquid is determined by the channel dimensions between junction J5 and J6. Typically, the volume of combined neutralization liquid is about the same as the volume of combined release liquid. In some embodiments, the volume of liquid positioned between junctions J5 and J6 is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions J5 and J6 is about 2.25 microliters (e.g., the total volume of release liquid and neutralization buffer is about 4 microliters).

Returning to FIGS. 6A, 6B, the combined release liquid and neutralization buffer move along mixing channel C15 and into channel C32 (vented downstream by air vent AV8). Motion continues until the upstream interface of the combined liquids passes hydrophobic vent H11, which vents pressure from actuator P4 stopping further motion of the combined liquids.

Continuing with operation of device 300, actuator P5 and gates G14, G15 and G17 are actuated to dissolve the lyophilized PCR particle present in second processing region 132 in water from reagent reservoir R4. Hydrophobic vent H10 vents pressure from actuator P5 upstream of the water stopping further motion. Dissolution of a PCR-reagent pellet typically occurs in about 2 minutes or less (e.g., in about 1 minute or less). Valve V17 is closed.

Continuing with operation of device 300, actuator P6 and gate G16 are actuated to drive the dissolved compounds of the lyophilized particle from processing region B2 into channel C31, where the dissolved reagents mix to form a homogenous dissolved lyophilized particle solution. Actuator P6 moves the solution into channels C35 and C33 (vented downstream by air vent AV5). Hydrophobic vent H9 vents pressure generated by actuator P6 upstream of the solution stopping further motion. Valves V18, V19, V20', and V22' are closed.

Continuing with operation of device 300, actuator P7 and gates G18, MG20 and G22 are actuated to combine (e.g., mix) a portion of neutralized release liquid in channel 32 between gate MG20 and gate G22 and a portion of the dissolved lyophilized particle solution in channel C35 between gate G18 and MG20. The combined liquids travel along a mixing channel C37 and into detection region D2. An air vent AV3 vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent H13, the pressure from actuator P7 is vented and the combined liquids are positioned within detection region D2.

Actuator P8 and gates MG2, G23, and G19 are actuated to combine a portion of water from reagent reservoir R4 between MG2 and gate G23 with a second portion of the dissolved lyophilized particle solution in channel C33 between gate G19 and MG2. The combined liquids travel along a mixing channel C41 and into detection region D1. An air vent AV4 vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent 1112, the pressure

from actuator P8 is vented and the combined liquids are positioned within detection region D1.

Continuing with operation of device 300, double valves V26' and V27' are closed to isolate detection region D1 from network 304 and double valves V24' and V25' are closed to isolate detection region D2 from network 304. The contents of each detection region (neutralized release liquid with sample polynucleotides in detection region D2 with PCR reagents from dissolved lyophilized particle solution and deionized water with PCR reagents from dissolved lyophilized particle solution in detection region D1) are subjected to heating and cooling steps to amplify polynucleotides (if present in detection region D2). The double valves of each detection region prevent evaporation of the detection region contents during heating. The amplified polynucleotides are typically detected using fluorescence detection.

While reservoirs have been shown as having a piercing member formed of a wall of the reservoir, other configurations are possible. For example, in some embodiments, the reservoir includes a needle-like piercing member that extends through an upper wall of the reservoir into the sealed space toward a lower wall of the reservoir. The upper wall of the reservoir may be sealed at the needle-like piercing member (e.g., with an adhesive, an epoxy). In use, the upper wall is depressed driving the piercing member through the lower wall forcing liquid in the sealed space to enter a microfluidic network.

While reservoirs have been described as including an actuation mechanism (e.g., a piercing member), other configurations are possible. For example, in some embodiments, a lower wall of the sealed space of the reservoir includes a weakened portion that overlies an opening to a microfluidic network. The lower wall material (e.g., laminate, polymer film, or foil) that overlies the opening is thick enough to prevent loss of the liquid within the sealed space but thin enough to rupture upon the application of pressure to the liquid therein. Typically, the material overlying the opening is thinner than the adjacent material. Alternatively, or in addition, the weakened material can be formed by leaving this material relatively unsupported as compared to the surrounding material of the lower wall.

While reservoirs have been described as having a sealed spaced formed in part by a wall of the sealed space, other configurations are possible. For example, referring to FIG. 11A, a reservoir includes a plunger-like actuation mechanism (e.g., a piercing member 342) and a gasket-like sealed space 343 having upper and lower layers 344, 345 respectively (e.g., upper and lower laminate layers). Liquid is sealed between the upper and lower layers. The sealed space can be surrounded by a supporting structure 346 (e.g., a toroidal gasket) that supports the sealed space at its upper and lower peripheral surfaces.

Referring to FIG. 11B, piercing member 342 is shown as being depressed until the piercing member 342 has pierced both the upper and lower layers bringing the liquid into communication with the microfluidic network. A vent 346 adjacent the plunger allows gas trapped between the piercing member and the upper layer of the sealed space to escape without being forced into the microfluidic network.

Referring to FIG. 11C, piercing member 342 is shown as fully actuated. A portion of the piercing member has displaced a corresponding volume of liquid from the sealed space and introduced the predetermined volume of liquid into the microfluidic device.

While the reservoirs have been described as having a sealed space that may be stationary with respect to a piercing

member, other configurations are possible. For example, FIG. 12A illustrates a reservoir having a sealed space 347 that is secured with (e.g., integral with) respect to an actuation mechanism having a movable member 348 (e.g., a plunger) and a piercing member 349 supported by a piercing member support 350 that are stationary with respect to the sealed space. Typically, the sealed space is defined by a cavity within the movable member and a lower wall 351 that seals liquid within the sealed space. Piercing member is configured to rupture the lower wall when the movable member is depressed. Piercing member support has a shape generally complementary to the cavity of the movable member. Piercing member support includes a channel 352 connected to a microfluidic network to allow fluid released from the enclosed space to enter the microfluidic network.

Referring to FIG. 12B, the movable member has been depressed so that the piercing member has just ruptured the lower layer of the sealed space. Referring to FIG. 12C, the reservoir has been fully depressed onto the piercing member and piercing member support. The volume of fluid displaced from the reservoir generally corresponds to the volume of the piercing member support that enters the enclosed space. A channel 353 allows air displaced by the moveable member to exit.

While reservoirs have been described as having a piercing member that is secured with respect to some portion of the reservoir, other configurations are possible. For example, referring to FIG. 13, a reservoir includes an actuation mechanism 354 (e.g., a piercing member such as a needle-like piercing member) that is unsecured with respect to the reservoir. A sealed space 355 of the reservoir is defined by an upper wall 356 and includes a channel 357 extending through a portion of a substrate 361 in which a microfluidic network is defined. A lower wall 358 of the sealed space separates the sealed space from a channel 359 of the microfluidic network. The piercing member occupies the channel 357 of the sealed space so that the piercing tip 360 of the piercing member rests against the lower wall 358. Depressing the upper wall 356 of the reservoir drives the piercing member 354 through the lower wall and forces liquid within the sealed space into the microfluidic network.

As another example, FIGS. 14A and 14B illustrate a reservoir including an actuation mechanism (e.g., a piercing member) that is initially secured to an interior of an upper wall of the reservoir but separates at least partially from the upper wall upon actuation of the reservoir.

As yet another example, FIGS. 15A and 15B illustrate a reservoir including a piercing member 364 that is initially secured to an interior 365 of an upper wall 366 of the reservoir but substantially separates (e.g., completely separates) from the upper wall upon actuation of the reservoir.

While reservoirs have been described as having an enclosed space that is fixed or otherwise integral with a portion of the reservoir, other configurations are possible. For example, referring to FIG. 16, a reservoir includes a capsule-like enclosed space 367 defined by an outer wall 368. The outer wall is generally formed of a material having a low vapor transmission rate. Reservoir also includes an actuation mechanism having a moveable member 369 with a piercing member 370 that pierces the enclosed space to release liquid therein. The liquid passes along a channel 372 leading to a microfluidic network. A channel 371 allows gas (e.g., air) otherwise trapped by the moveable member to exit.

While reservoirs have been described as generally overlying an inlet to a microfluidic network, other configurations are possible. For example, referring to FIG. 17, a reservoir includes an enclosed space 373 in which liquid is stored and

US 10,604,788 B2

27

a connecting portion 374 connected to an inlet 376 of a microfluidic network. The enclosed space 373 and connecting portion 374 are separated by a rupturable seal 375 (e.g., a weak seal). In general, the rupturable seal 375 prevents liquid or vapor from exiting the enclosed space. However, upon the application of pressure to the liquid (e.g., by depressing a wall 377 of the enclosed space), the rupturable seal 375 ruptures allowing the liquid to pass through the weak seal to the connecting portion and into the microfluidic network 378.

A still further embodiment of a reservoir with a piercing member is shown in FIG. 27A, which shows a reservoir 2701 having an outer shell 2703 and a piercing element 2704 that are both made of the same piece of material. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element 2704 is generally conical in shape, with the apex adjacent to a membrane 2702; its apex preferably does not exceed 0.040". The piercing element will puncture membrane 2702 and release liquid from reservoir 2701 when the outer shell is depressed. Representative dimensions are shown on FIG. 27A. The reservoir may be constructed so that the upper surface is level, with a flat protective piece 2705 covering the base of the conical shape of piercing element 2704.

Yet another embodiment of a reservoir with a piercing member is shown in FIG. 27B, showing a reservoir 2711 having a single-piece outer shell 2712 and piercing element 2714. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thereto-forming and injection moulding. Piercing element 2714 can be frustoconical in shape, with its narrower side adjacent to membrane 2713. Alternatively, piercing element 2714 can comprise several separate piercing elements, arranged within a conical space. Preferably there are four such piercing elements where multiple elements are present.

It is to be understood that the dimensions of the reservoir, piercing element, shell and moulding shown in FIGS. 27A and 27B as decimal quantities in inches are exemplary. In particular, the dimensions are such that the shell does not collapse under its own weight and is not so as strong to prohibit depression of the piercing member when required during operation of the device.

Furthermore, the materials of the various embodiments are also chosen so that the device has a shelf-life of about a year. By this it is meant that the thickness of the various materials are such that they resist loss, through means such as diffusion, of 10% of the liquid volume contained therein over a desired shelf-life period.

Preferably the volume of the reservoir is around 150 μ L before a shell is depressed. Upon depression of a shell, the volume is preferably deformed to around half its original volume.

While devices for processing samples have been described as having a generally planar configuration, other configurations can be used. For example, referring to FIG. 18, a device 700 configured to process a polynucleotide-containing sample, such as to prepare the sample for amplification of the polynucleotides, has a generally tube-like or vial-like configuration. Device 700 includes a sample reservoir 704, a reagent reservoir 706, a gas pressure generator 708, a closure (e.g., a cap 710), and a processing region 702 including a retention member 704 having a plurality of particles (e.g. carboxylate beads 705 surface-modified with a ligand, e.g., poly-L-lysine and/or poly-D-lysine, or poly-

28

ethyleneimine). Retention member 705 and beads 705 may share any or all properties of retention member 216 and surface-modified particles 218. Device 700 also includes an opening 716 and a valve, e.g., a thermally actuated valve 714 for opening and closing opening 716.

In use, a polynucleotide-containing sample is added to sample reservoir 704. Typical sample amounts range from about 100 μ L to about 2 mL, although greater or smaller amounts may be used.

Reagent reservoir 706 may be provided to users of device 700 with pre-loaded reagent. Alternatively, device 700 may be configured so that users add reagent to device 700. In any event, the reagents may include, e.g., NaOH solutions and/or buffer solutions such as any of such solutions discussed herein.

Once sample and, if necessary, reagent have been added to device 700, cap 710 is closed to prevent evaporation of sample and reagent materials.

Referring also to FIG. 19, an operator 718 is configured to operate device 700. Operator 718 includes a first heat source 720 and a second heat source 722. First heat source 720 heats sample present within sample reservoir 704, such as to lyse cells of the polynucleotide-containing sample to prepare free polynucleotides.

Device 700 may also include an enzyme reservoir 712 comprising an enzyme, e.g., a protease such as pronase, configured to cleave peptide bonds of polypeptides present in the polynucleotide-containing sample. Enzyme reservoir 712 may be provided to users of device 700 with pre-loaded enzyme. Alternatively, device 700 may be configured so that users add enzyme to device 700.

Device 700 may be used to reduce the amount of inhibitors present relative to the amount of polynucleotides to be determined. Thus, the sample is eluted through processing region 702 to contact constituents of the sample with beads 705. Beads 705 retain polynucleotides of the sample as compared to inhibitors as described elsewhere herein. With valve 714 in the open state, sample constituents not retained in processing region 702 exit device 700 via the opening.

Once the polynucleotide-containing sample has eluted through processing region 702, an amount of reagent, e.g., a wash solution, e.g., a buffer such as Tris-EDTA pH 8.0 with 1% Triton X 100 is eluted through processing region 702. The wash solution is generally stored in reagent reservoir 706, which may include a valve configured to release an amount of wash solution. The wash solution elutes remaining polynucleotide-containing sample and inhibitors without eluting retained polynucleotides.

Once inhibitors have been separated from retained polynucleotides, the polynucleotides are released from beads 705. In some embodiments, polynucleotides are released by contacting the beads 705 with a release solution, e.g., a NaOH solution or buffer solution having a pH different from that of the wash solution. Alternatively, or in combination, beads 705 with retained polynucleotides are heated, such as by using second heat source 722 of operator 718. When heat is used to release the polynucleotides, the release solution may be identical with the wash solution.

Gas pressure generator 708 may be used to expel an amount of release solution with released polynucleotides from device 700. Gas pressure generator and/or operator 718 may include a heat source to heat gas present within generator 708. The heated gas expands and provides the gas pressure to expel sample. In some embodiments, and whether or not thermally generated gas pressure is used, gas pressure generator 708 is configured to expel a predetermined volume of material. Typically, the amount of expelled

US 10,604,788 B2

29

solution is less than about 500 μL , less than about 250 μL , less than about 100 μL , less than about 50 μL , e.g., less than about 25 μL .

EXAMPLES

The following Examples are illustrative and are not intended to be limiting.

Example 1 Preparing Retention Member

Carboxylate surface magnetic beads (Sera-Mag Magnetic Carboxylate modified, Part #3008050250, Seradyn) at a concentration of about 1011 mL⁻¹ were activated for 30 minutes using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in a pH 6.1 500 mM 2-(N-Morpholinio)-ethanesulfonic acid (MES) buffer solution. Activated beads were incubated with 3000 Da or 300,000 Da average molecular weight poly-L-lysine (PLL). After 2 washes to remove unbound PLL, beads were ready for use.

Example 2 Microfluidic Device

Referring to FIGS. 20 and 21, a microfluidic device 300 was fabricated to demonstrate separation of polynucleotides from inhibitors. Device 300 comprises first and second substrate portions 302', 304', which respectively comprise first and second layers 302a', 302b' and 304a', 304b'. First and second layers 302a', 302b' define a channel 306' comprising an inlet 310' and an outlet 312'. First and second layers 304a', 304b' define a channel 308' comprising an inlet 314' and an outlet 316'. First and second substrate portions 302', 304' were mated using adhesive 324' so that outlet 312' communicated with inlet 314' with a filter 318' positioned therebetween. A portion of outlet 312' was filed with the activated beads prepared above to provide a processing region 320' comprising a retention member (the beads). A pipette 322' (FIG. 22) secured by adhesive 326' facilitated sample introduction.

In use, sample introduced via inlet 310' passed along channel and through processing region 320'. Excess sample material passed along channel 308' and exited device 300' via outlet 316'. Polynucleotides were preferentially retained by the beads as compared to inhibitors. Once sample had been introduced, additional liquids, e.g., a wash liquid and/or a liquid for use in releasing the retained polynucleotides were introduced via inlet 326'.

Example 3 Retention of DNA

Retention of polynucleotides by the poly-L-lysine modified beads of device 300' was demonstrated by preparing respective devices comprising processing regions having a volume of about 1 μL including about 1000 beads. The beads were modified with poly-L-lysine of between about 15,000 and 30,000 Da. Each processing region was filled with a liquid comprising herring sperm DNA (about 20 μL of sample with a concentration of about 20 mg/mL) thereby placing the beads and liquid in contact. After the liquid and beads had been in contact for 10 minutes, the liquid was removed from each processing region and subjected to quantitative real-time PCR to determine the amount of herring sperm DNA present in the liquid.

Two controls were performed. First, an otherwise identical processing region was packed with unmodified beads, i.e., beads that were identical with the poly-L-lysine beads

30

except for the activation and poly-L-lysine incubation steps. The liquid comprising herring sperm DNA was contacted with these beads, allowed to stand for 10 minutes, removed, and subjected to quantitative real-time PCR. Second, the liquid comprising the herring sperm DNA ("the unprocessed liquid") was subjected to quantitative real-time PCR.

Referring to FIG. 22, the first and second controls exhibited essentially identical responses indicating the presence of herring sperm DNA in the liquid contacted with the unmodified beads and in the unprocessed liquid. The liquid that had contacted the 3,000 poly-L-lysine beads exhibited a lower response indicating that the modified beads had retained substantially all of the herring sperm DNA. The PCR response of the liquid that had contacted the 300,000 Da poly-L-lysine beads exhibited an amplification response that was at least about 50% greater than for the 3,000 Da beads indicating that the lower molecular weight surface modification was more efficient at retaining the herring sperm DNA.

Example 4 Releasing DNA From Poly-L-Lysine Modified Beads

Devices having processing regions were packed with 3,000 Da poly-L-lysine modified beads. Liquid comprising polynucleotides obtained from group B streptococci (GBS) was contacted with the beads and incubated for 10 minutes as above for the herring sperm DNA. This liquid had been obtained by subjecting about 10,000 GBS bacteria in 10 μL of 20 mM Tris pH 8, 1 mM EDTA, 1% Triton X-100 buffer to thermal lysing at 97° C. for 3 min.

After 10 minutes, the liquid in contact with the beads was removed by flowing about 10 μL of wash solution (Tris-EDTA pH 8.0 with 1% Triton X 100) through the processing region. Subsequently, about 1 μL of 5 mM NaOH solution was added to the processing region. This process left the packed processing region filled with the NaOH solution in contact with the beads. The solution in contact with the beads was heated to 95° C. After 5 minutes of heating at 95° C., the solution in contact with the beads was removed by eluting the processing region with a volume of solution equal to three times the void volume of the processing region.

Referring to FIG. 23, five aliquots of solution were subjected to quantitative real-time PCR amplification. Aliquots E1, E2, and E3 each contained about 1 μL of liquid. Aliquot L corresponds to liquid of the original sample that had passed through the processing region. Aliquot W was liquid obtained from wash solution without heating. Aliquot E1 corresponds to the dead volume of device 300, about equal to the volume of channel 308. Thus, liquid of aliquot E1 was present in channel 308 and not in contact with the beads during heating. This liquid had passed through the processing region prior to heating. Aliquot E2 comprises liquid that was present within the processing region and in contact with the beads during heating. Aliquot E3 comprises liquid used to remove aliquot E2 from the processing region.

As seen in FIG. 23, more than 65% of the GBS DNA present in the initial sample was retained by and released from the beads (Aliquot E2). Aliquot E2 also demonstrates the release of more than 80% of the DNA that had been retained by the beads. Less than about 18% of the GBS DNA passed through the processing region without being cap-

US 10,604,788 B2

31

tured. The wash solution without heating comprised less than 5% of the GBS DNA (Aliquot W).

Example 5 Separation of Polynucleotides and Inhibitors

Buccal cells from the lining of the cheeks provide a source of human genetic material (DNA) that may be used for single nucleotide polymorphism (SNP) detection. A sample comprising buccal cells was subjected to thermal lysing to release DNA from within the cells. Device 300 was used to separate the DNA from concomitant inhibitors as described above. A cleaned-up sample corresponding to aliquot E2 of FIG. 23 was subjected to polymerase chain reaction. A control or crude sample as obtained from the thermal lysing was also amplified.

Referring to FIG. 24, the cleaned-up sample exhibited substantially higher PCR response in fewer cycles than did the control sample. For example, the clean-up sample exceeded a response of 20 within 32 cycles whereas the control sample required about 45 cycles to achieve the sample response.

Blood acts as a sample matrix in variety of diagnostic tests including detection of infectious disease agents, cancer markers and other genetic markers. Hemoglobin present in blood samples is a documented potent inhibitor of PCR. Two 5 ml blood samples were lysed in 20 mM Tris pH 8, 1 mM EDTA, 1% SDS buffer and introduced to respective devices 300, which were operated as described above to prepare two clean-up samples. A third 5 ml blood sample was lysed and prepared using a commercial DNA extraction method Puregene, Gentra Systems, MN. The respective cleaned-up samples and sample subjected to the commercial extraction method were used for a Allelic discrimination analysis (CYP2D6*4 reagents, Applied Biosystems, CA). Each sample contained an amount of DNA corresponding to about 1 ml of blood.

Referring to FIG. 25, the cleaned-up and commercially extracted samples exhibited similar PCR response demonstrating that the processing region of device 300' efficiently removed inhibitors from the blood samples.

Example 6 Protease Resistant Retention Member

The preparation of polynucleotide samples for farther processing often includes subjecting the samples to protease treatment in which a protease cleaves peptide bonds of proteins in the sample. An exemplary protease is pronase, a mixture of endo- and exo-proteases. Pronase cleaves most peptide bonds. Certain ligands, such as poly-L-lysine are susceptible to rupture by pronase, and other proteases. Thus, if samples are generally not subjected to protease treatment in the presence of the retention member if the ligands bound thereto are susceptible to the proteases.

Poly-D-lysine, the dextro enantiomer of poly-lysine resists cleavage by pronase and other proteases. The ability of a retention member comprising bound poly-D-lysine to retain DNA even when subjected to a protease treatment was studied.

Eight (8) samples were prepared. A first group of 4 samples contained 1000 GBS cells in 10 μ l buffer. A second group of 4 samples contained 100 GBS cells in 10 μ l buffer. Each of the 8 samples was heated to 97° C. for 3 min to lyse the GBS cells. Four (4) sample sets were created from the heated samples. Each sample set contained 1 sample from each of the first and second groups. The samples of each sample sets were treated as follows.

32

Referring to FIG. 26A, the samples of sample set 1 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 2 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 3 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 4 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

As seen in FIG. 26B, an average of more than 80% of DNA from the GBS cells was recovered using sample set 4 in which the samples were contacted with poly-D-lysine modified beads and subjected to pronase incubation in the presence of the beads without protease inactivation. The

US 10,604,788 B2

33

recovery efficiency for sample set 4 is more than twice as high as for any of the other samples. Specifically, the recovery efficiencies for sample sets 1, 2, 3, and 4, were 29%, 32%, 14%, and 81.5%, respectively. The efficiencies demonstrate that high recovery efficiencies can be obtained for samples subjected to protease incubation in the presence of a retention member that retains DNA.

Other embodiments are within the claims.

What is claimed is:

1. A system for processing polynucleotides in a biological sample, the system comprising:

a microfluidic device comprising substrate layers that define a microfluidic network, the microfluidic network comprising a first processing region, the microfluidic device further comprising a waste chamber downstream of the first processing region;

a lysing container located external to the substrate layers, wherein the lysing container is configured to receive the biological sample and configured to place the biological sample in contact with a lysing reagent to release polynucleotides from the biological sample into a lysate solution;

a plurality of magnetic binding particles disposed in the lysing container, the plurality of magnetic binding particles comprising polycationic molecules on the surfaces thereof, wherein the plurality of magnetic binding particles are configured to retain at least a portion of the polynucleotides on the surface thereof in the lysate solution at a pH of 8.5 or less;

a first heat source and a second heat source located external to the microfluidic network;

a lysing heater configured to apply heat to the lysate solution and the plurality of magnetic binding particles in the lysing container, wherein the lysing heater is separate and distinct from the first and second heat sources;

an operating system comprising a processor, the processor configured to actuate the first and second heat sources and the lysing heater;

wherein the operating system is configured to actuate the lysing heater to heat the lysate solution in the lysing container to a first temperature;

wherein the first processing region of the microfluidic network is configured to receive the lysate solution and the plurality of magnetic binding particles from the lysing container, wherein the waste chamber is configured to receive excess lysate solution downstream of the first processing region as the plurality of magnetic binding particles are retained in the first processing region;

wherein the microfluidic network is configured to receive a wash solution in the first processing region to remove unbound material not retained by the plurality of magnetic binding particles;

a release solution having a pH of at least 11.4;

wherein the microfluidic network further comprises an inlet for receiving the release solution into the microfluidic network and one or more channels leading from the inlet to the first processing region;

wherein the first processing region is configured to receive the release solution therein, and wherein, in the presence of the release solution in the first processing region, the plurality of magnetic binding particles are configured to release at least a portion of the polynucleotides into an eluate solution in the first processing region;

34

wherein the first heat source is configured to apply heat to the lysate solution and the plurality of magnetic binding particles in the first processing region, and wherein the operating system is configured to actuate the first heat source to heat the lysate solution in the first processing region to a second temperature greater than the first temperature; and

a second processing region comprising PCR reagents, the second processing region configured to receive the eluate solution containing polynucleotides and configured to place the eluate solution in contact with PCR reagents to form a PCR-ready solution.

2. The system of claim 1, wherein the polycationic molecules are covalently bound to the surfaces of the plurality of magnetic binding particles, and wherein the plurality of magnetic binding particles comprise one or more carboxylic groups to provide an attachment point for the polycationic molecules.

3. The system of claim 1, wherein the release solution comprises NaOH.

4. The system of claim 3, wherein the release solution has a pH between about 11.7 and about 13.

5. The system of claim 1, wherein the plurality of magnetic binding particles are preloaded in the lysing container.

6. The system of claim 5, further comprising a proteinase K enzyme preloaded in the lysing container.

7. The system of claim 1, wherein the PCR reagents contained in the second processing region comprise primers and probes specific to group B *streptococcus* (GBS) bacteria.

8. The system of claim 1, wherein the PCR reagents in the second processing region are dry PCR reagents.

9. The system of claim 8, wherein the dry PCR reagents have a room-temperature shelf life of at least 12 months.

10. The system of claim 1, wherein the plurality of substrate layers comprise:

an injection molded layer; and

a laminate configured to be secured to another substrate layer to seal components of the microfluidic network.

11. The system of claim 1, wherein the operating system is configured to actuate the lysing heater to heat the lysate solution to between about 30° C. and about 50° C.

12. The system of claim 1, wherein the operating system is configured to actuate the lysing heater to heat the lysate solution to about 60° C.

13. The system of claim 1, wherein the operating system is configured to actuate the first heat source to heat the lysate solution to between about 80° C. and about 100° C.

14. The system of claim 1, wherein the operating system is configured to actuate the lysing heater to heat the contents of the lysing container for about 10 minutes.

15. The system of claim 1, wherein the polycationic molecules have a molecular weight of less than about 30,000 Da.

16. The system of claim 1, wherein the polycationic molecules have a molecular weight of less than about 800 Da.

17. The system of claim 1, further comprising an introduction device configured to apply air pressure to move the lysate solution through one or more channels in the microfluidic device into the first processing region.

18. The system of claim 17, wherein the introduction device is configured to apply air pressure through an inlet of the microfluidic device.

19. The system of claim 17, wherein the introduction device comprises a syringe or a pipette.

US 10,604,788 B2

35

20. The system of claim 1, further comprising an automated sampling device configured to remove the eluate solution from the first processing region.

21. The system of claim 1, further comprising a syringe or a pipette configured to remove the eluate solution from the first processing region.

22. A system comprising:

a lysing container configured to receive a biological sample;

a plurality of magnetic binding particles disposed in the lysing container, the plurality of magnetic binding particles comprising polycationic molecules on the surfaces thereof, the lysing container configured to place the biological sample in contact with a lysing reagent to release polynucleotides from the biological sample into a lysate solution, the plurality of magnetic binding particles configured to retain at least a portion of the polynucleotides on the surface thereof at a pH of about 8.5 or less in the lysate solution;

substrate layers defining a microfluidic network that comprises a plurality of microfluidic components including a first processing region, wherein the first processing region is configured to receive, from the lysing container, the lysate solution and the plurality of magnetic binding particles retaining the polynucleotides on the surface thereof;

wherein the lysing container is located external to the substrate layers defining the microfluidic network;

a plurality of heat sources having locations that correspond to the plurality of microfluidic components of the microfluidic network, wherein in use, the microfluidic network is disposed in thermal contact with the plurality of heat sources;

wherein the plurality of heat sources are located external to the microfluidic network;

a heater spatially separate and distinct from the plurality of heat sources, the heater configured to heat the lysing container;

an operating system comprising a processor, the processor configured to actuate the plurality of heat sources and the heater;

a waste chamber, the waste chamber configured to receive a wash solution after the wash solution contacts the plurality of magnetic binding particles in the first processing region and removes compounds not retained by the plurality of magnetic binding particles;

a release solution having a pH of at least 11.4;

wherein the microfluidic network further comprises an inlet for receiving the release solution into the microfluidic network and one or more channels leading from the inlet to the plurality of magnetic binding particles retaining the polynucleotides on the surface thereof in the lysate solution in the first processing region;

wherein the plurality of magnetic binding particles are configured to release at least a portion of the polynucleotides into an eluate solution when in the presence of the release solution in the first processing region and when heat is applied by a heat source of the plurality of heat sources to the lysate solution and the plurality of magnetic binding particles in the first processing region; and

a second processing region comprising PCR reagents and configured to receive the eluate solution containing the eluted polynucleotides to reconstitute the PCR reagents and form a PCR-ready solution.

36

23. The system of claim 22, wherein at least one of the plurality of heat sources comprises a resistive heat source operated by the processor.

24. The system of claim 22, wherein the substrate layers comprise:

an injection molded layer; and

a laminate configured to be secured to another substrate layer to seal components of the microfluidic network.

25. The system of claim 22, further comprising a release solution reservoir, wherein the release solution reservoir is located external to the substrate layers defining the microfluidic network.

26. The system of claim 22, wherein the polycationic molecules are covalently bound to the surfaces of the plurality of magnetic binding particles, and wherein the plurality of magnetic binding particles comprise one or more carboxylic groups to provide an attachment point for the polycationic molecules.

27. The system of claim 22, wherein the release solution comprises NaOH and has a pH between about 11.7 and about 13.

28. The system of claim 22, wherein the plurality of magnetic binding particles are preloaded in the lysing container.

29. The system of claim 28, further comprising a proteinase K enzyme preloaded in the lysing container.

30. The system of claim 22, wherein the PCR reagents in the processing region comprise primers and probes specific to group B streptococcus (GBS) bacteria.

31. The system of claim 22, wherein the PCR reagents in the second processing region are dry PCR reagents.

32. The system of claim 31, wherein the dry PCR reagents have a room-temperature shelf life of at least 12 months.

33. The system of claim 22, further comprising an introduction device configured to apply air pressure to the microfluidic network to move the lysate solution through one or more channels in the microfluidic network.

34. The system of claim 33, wherein the introduction device is configured to apply air pressure through an inlet of the microfluidic network.

35. The system of claim 34, wherein the introduction device comprises a syringe or a pipette.

36. The system of claim 22, further comprising an automated sampling device configured to remove the eluate solution from the microfluidic network.

37. The system of claim 22, further comprising a syringe or a pipette configured to remove the eluate solution from the microfluidic network.

38. The system of claim 22, further comprising an operating system configured to actuate the heater to heat the lysate solution in the lysing container to a temperature between about 30° C. and about 50° C.

39. The system of claim 22, further comprising an operating system configured to actuate the heater to heat the lysate solution in the lysing container to a temperature of about 60° C.

40. A system for processing polynucleotides from a biological sample, the system comprising:

a microfluidic network disposed in a plurality of substrate layers, wherein the microfluidic network comprises a processing region and a detection region;

a lysing container located external to the substrate layers, wherein the lysing container is configured to receive the biological sample and configured to place the biological sample in contact with a lysing reagent to release polynucleotides from the biological sample into a lysate solution;

US 10,604,788 B2

37

a plurality of magnetic binding particles disposed in the lysing container, the plurality of magnetic binding particles comprising polycationic molecules on the surfaces thereof, wherein the plurality of magnetic binding particles are configured to retain at least a portion of the polynucleotides on the surface thereof in the lysate solution at a pH of about 8.5 or less;

a first heat source and a second heat source, wherein in use, the processing region of the microfluidic network is disposed in thermal contact with the first heat source and the detection region is disposed in thermal contact with the second heat source;

wherein the first and second heat sources are located external to the microfluidic network;

a heater spatially separate and distinct from the first and second heat sources, the heater configured to heat the lysing container;

wherein the processing region of the microfluidic network is configured to receive the lysate solution and the plurality of magnetic binding particles from the lysing container;

wherein the microfluidic network is configured to receive a wash solution in the processing region to remove unbound material not retained by the plurality of magnetic binding particles;

a release solution having a pH of at least 11.4;

wherein the microfluidic network further comprises an inlet configured to receive the release solution into the microfluidic network and one or more channels leading from the inlet to the processing region, wherein the plurality of magnetic binding particles are configured to release at least a portion of the polynucleotides into an eluate solution in the presence of the release solution and heat applied to the lysate solution and the plurality of magnetic binding particles;

wherein the first heat source is configured to apply heat to the lysate solution and the plurality of magnetic binding particles in the processing region; and

wherein the detection region is configured to receive the eluate solution containing the at least a portion of the polynucleotides and dissolved PCR reagents.

41. The system of claim 40, further comprising a processor, wherein at least one of the first and second heat sources comprises a resistive heat source operated by the processor.

42. The system of claim 40, further comprising an operating system configured to actuate the heater to heat the lysing container for about 10 minutes.

43. The system of claim 40, wherein the plurality of substrate layers comprise:

38

an injection molded layer; and

a laminate configured to be secured to another substrate layer to seal components of the microfluidic network.

44. The system of claim 40, further comprising:

a release solution reservoir, wherein the release solution reservoir is located external to the microfluidic network; and

a buffer solution reservoir, wherein the buffer solution reservoir is located external to the microfluidic network.

45. The system of claim 40, wherein the polycationic molecules are covalently bound to the surfaces of the plurality of magnetic binding particles, and wherein the plurality of magnetic binding particles comprise one or more carboxylic groups to provide an attachment point for the polycationic molecules.

46. The system of claim 40, wherein the release solution comprises NaOH and has a pH between about 11.7 and about 13.

47. The system of claim 40, wherein the plurality of magnetic binding particles are preloaded in the lysing container.

48. The system of claim 47, further comprising a proteinase K enzyme preloaded in the lysing container.

49. The system of claim 40, wherein the PCR reagents comprise primers and probes specific to group B streptococcus (GBS) bacteria.

50. The system of claim 40, wherein the PCR reagents are dry prior to being dissolved and have a room-temperature shelf life of at least 12 months.

51. The system of claim 40, further comprising an introduction device configured to apply air pressure through an inlet of the microfluidic network to move the lysate solution through one or more channels into the processing region.

52. The system of claim 51, wherein the introduction device comprises a syringe or a pipette.

53. The system of claim 40, further comprising an operating system configured to actuate the heater to heat the lysate solution in the lysing container to a temperature between about 30° C. and about 50° C.

54. The system of claim 40, further comprising an operating system configured to actuate the heater to heat the lysate solution in the lysing container to a temperature of about 60° C.

55. The system of claim 40, further comprising an operating system configured to actuate the first heat source to heat the contents of the processing region to a temperature of between about 80° C. and about 100° C.

* * * * *

EXHIBIT 46



US010494663B1

(12) **United States Patent**
Wu et al.

(10) **Patent No.:** **US 10,494,663 B1**
(45) **Date of Patent:** ***Dec. 3, 2019**

(54) **METHOD FOR PROCESSING
POLYNUCLEOTIDE-CONTAINING SAMPLES**

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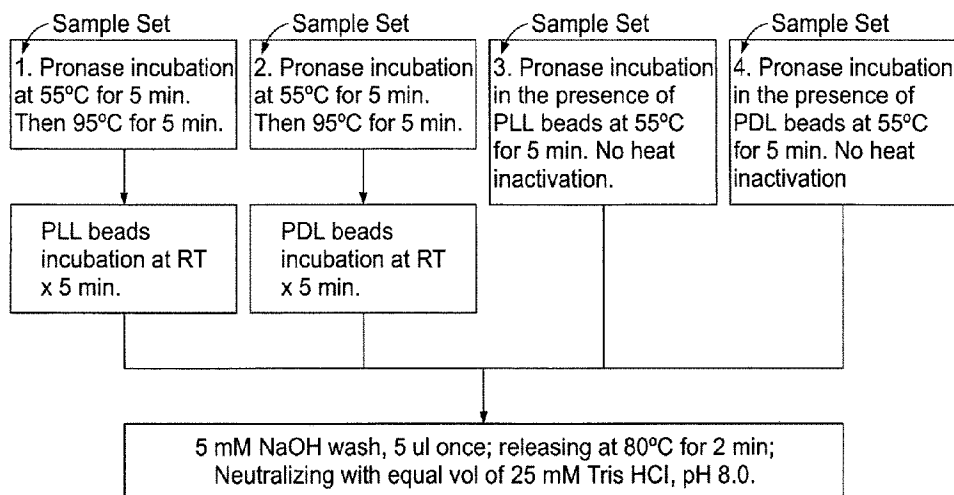
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(57) **ABSTRACT**

Methods and systems for processing polynucleotides (e.g.,
DNA) are disclosed. A processing region includes one or
more surfaces (e.g., particle surfaces) modified with ligands
that retain polynucleotides under a first set of conditions
(e.g., temperature and pH) and release the polynucleotides
under a second set of conditions (e.g., higher temperature
and/or more basic pH). The processing region can be used
to, for example, concentrate polynucleotides of a sample
and/or separate inhibitors of amplification reactions from the
polynucleotides. Microfluidic devices with a processing
region are disclosed.

49 Claims, 25 Drawing Sheets



US 10,494,663 B1

Page 2

Related U.S. Application Data

continuation of application No. 14/506,471, filed on Oct. 3, 2014, now Pat. No. 10,364,456, which is a continuation of application No. 11/281,247, filed on Nov. 16, 2005, now Pat. No. 8,852,862, which is a continuation-in-part of application No. PCT/US2005/015345, filed on May 3, 2005.

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US 10,494,663 B1

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US 10,494,663 B1

Page 7

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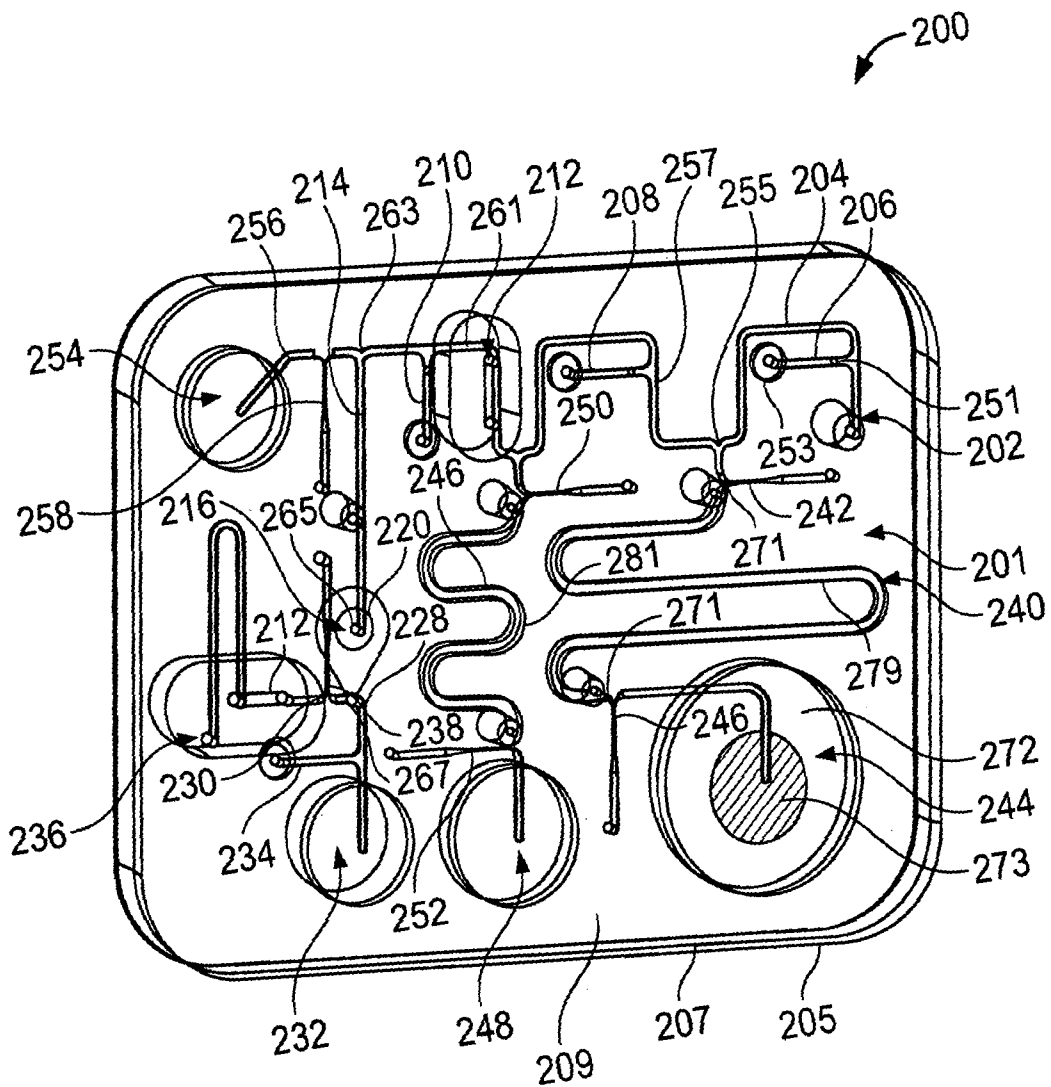


FIG. 1

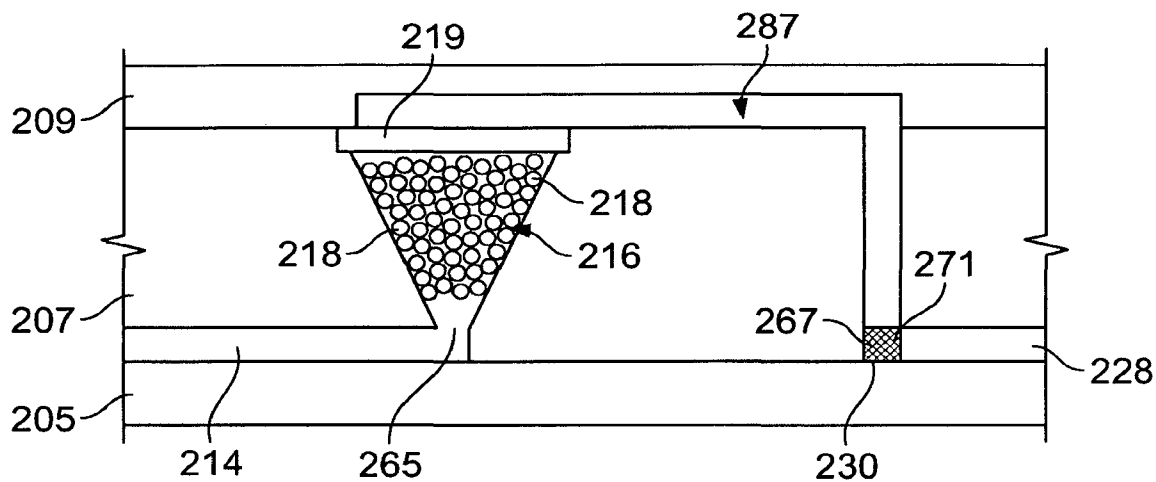


FIG. 2

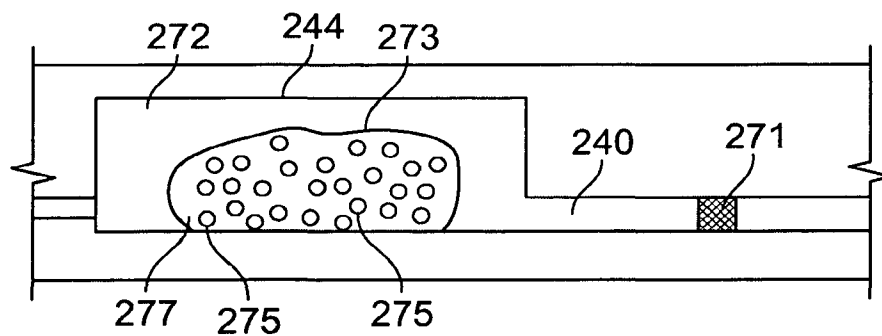


FIG. 3

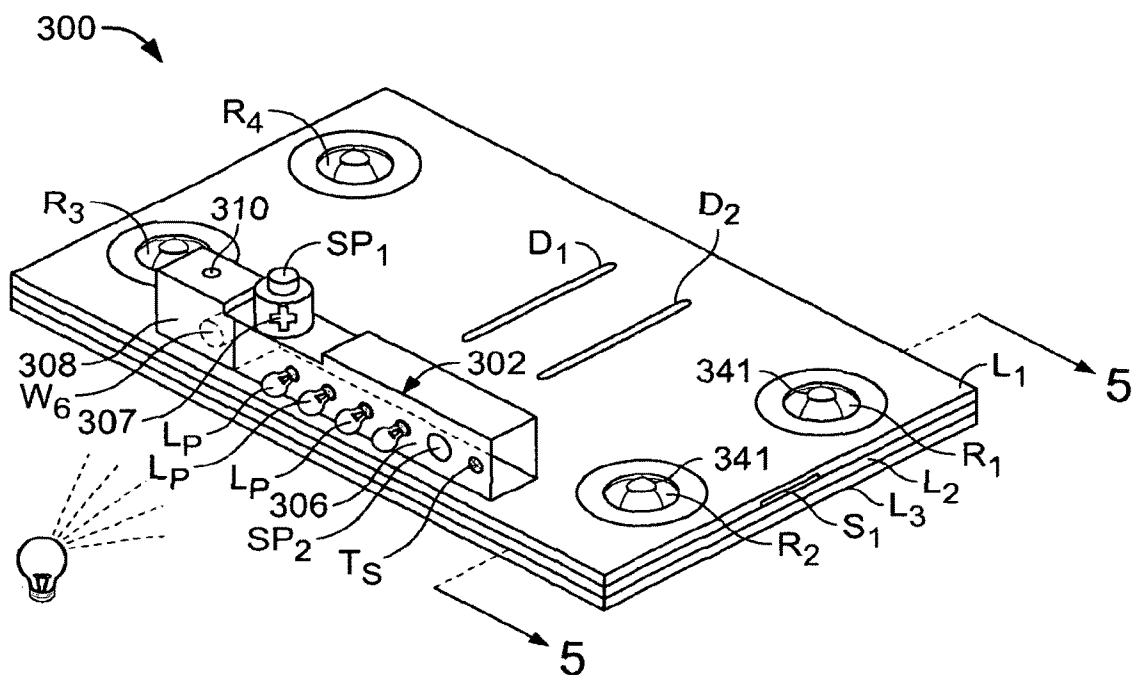


FIG. 4

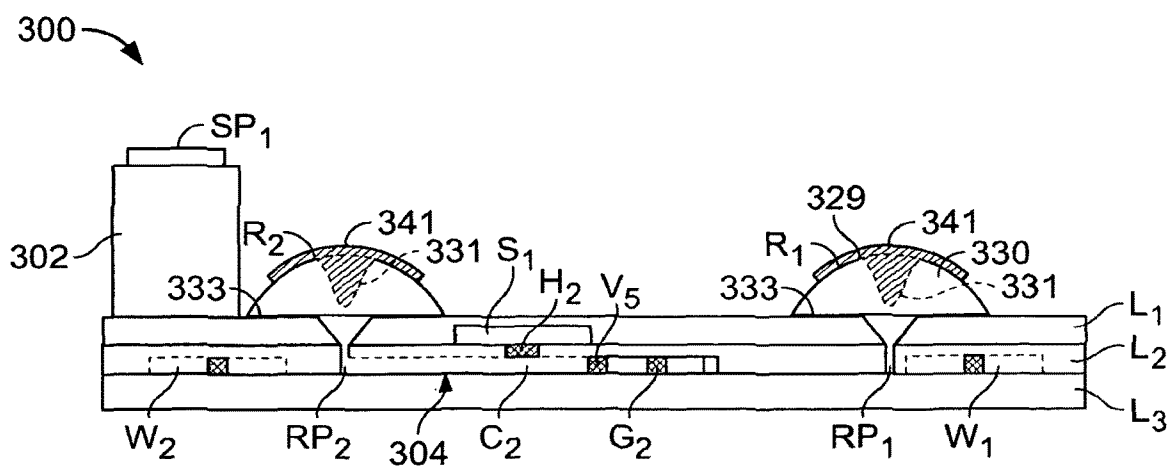


FIG. 5

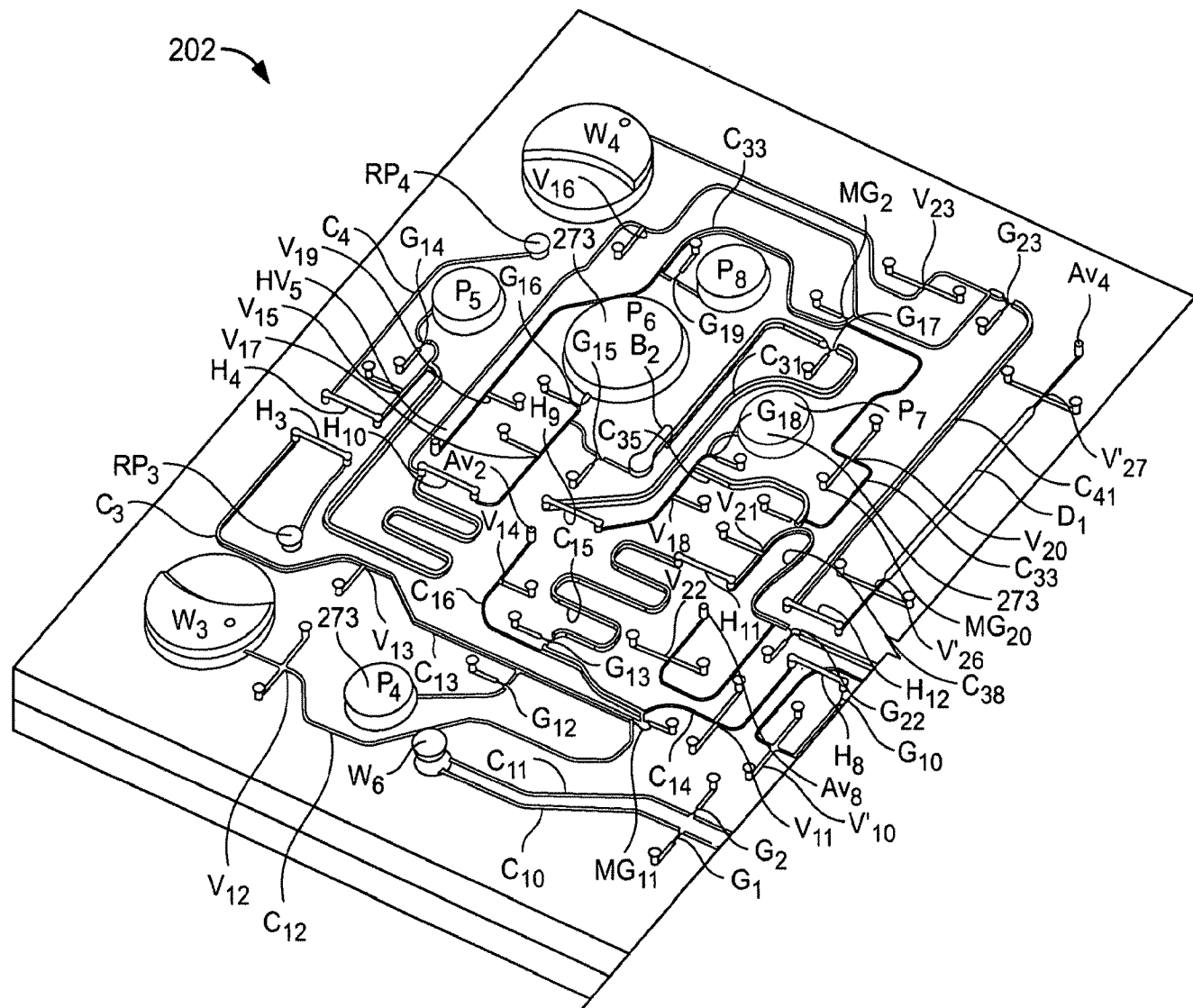


FIG. 6A

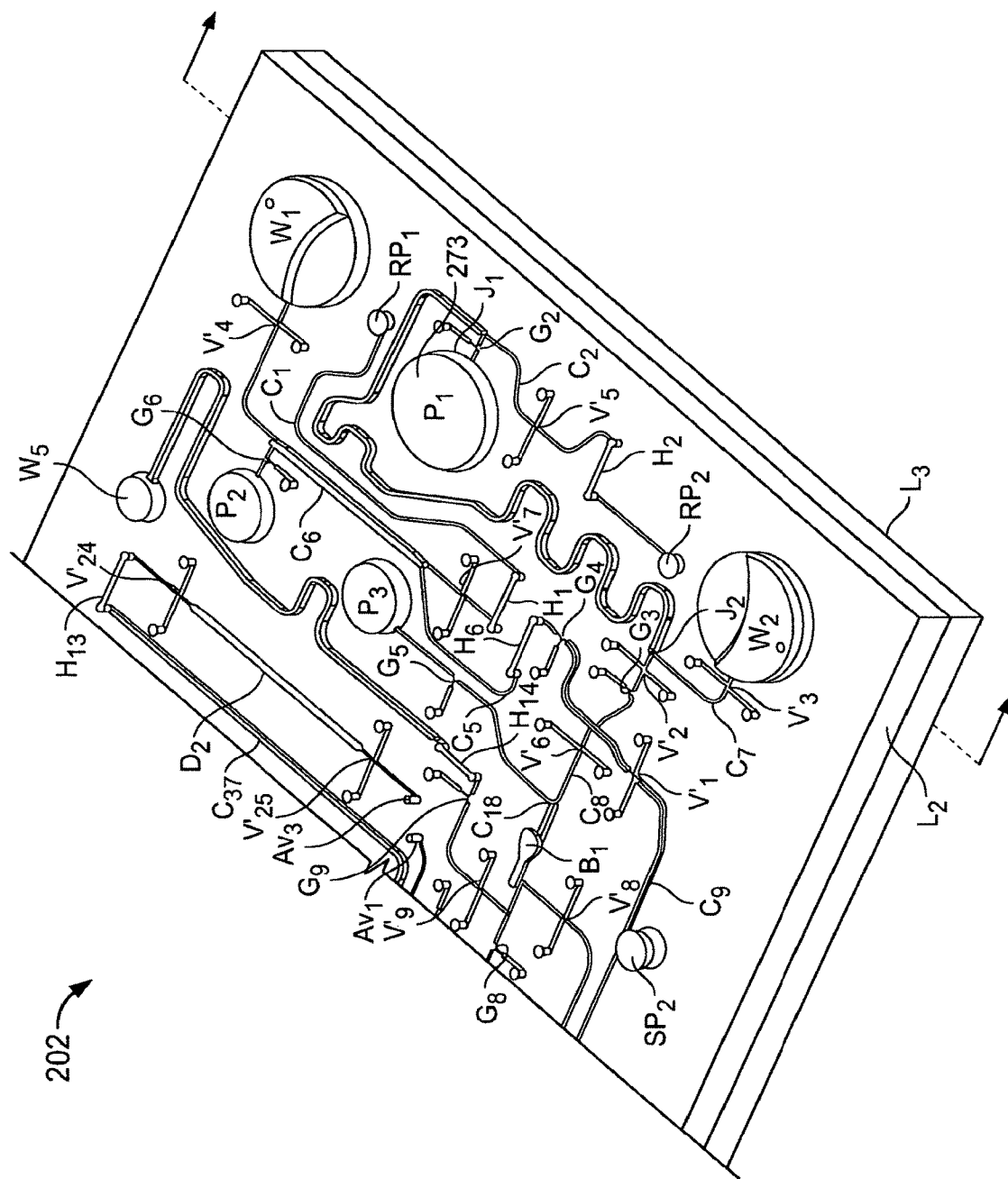


FIG. 6B

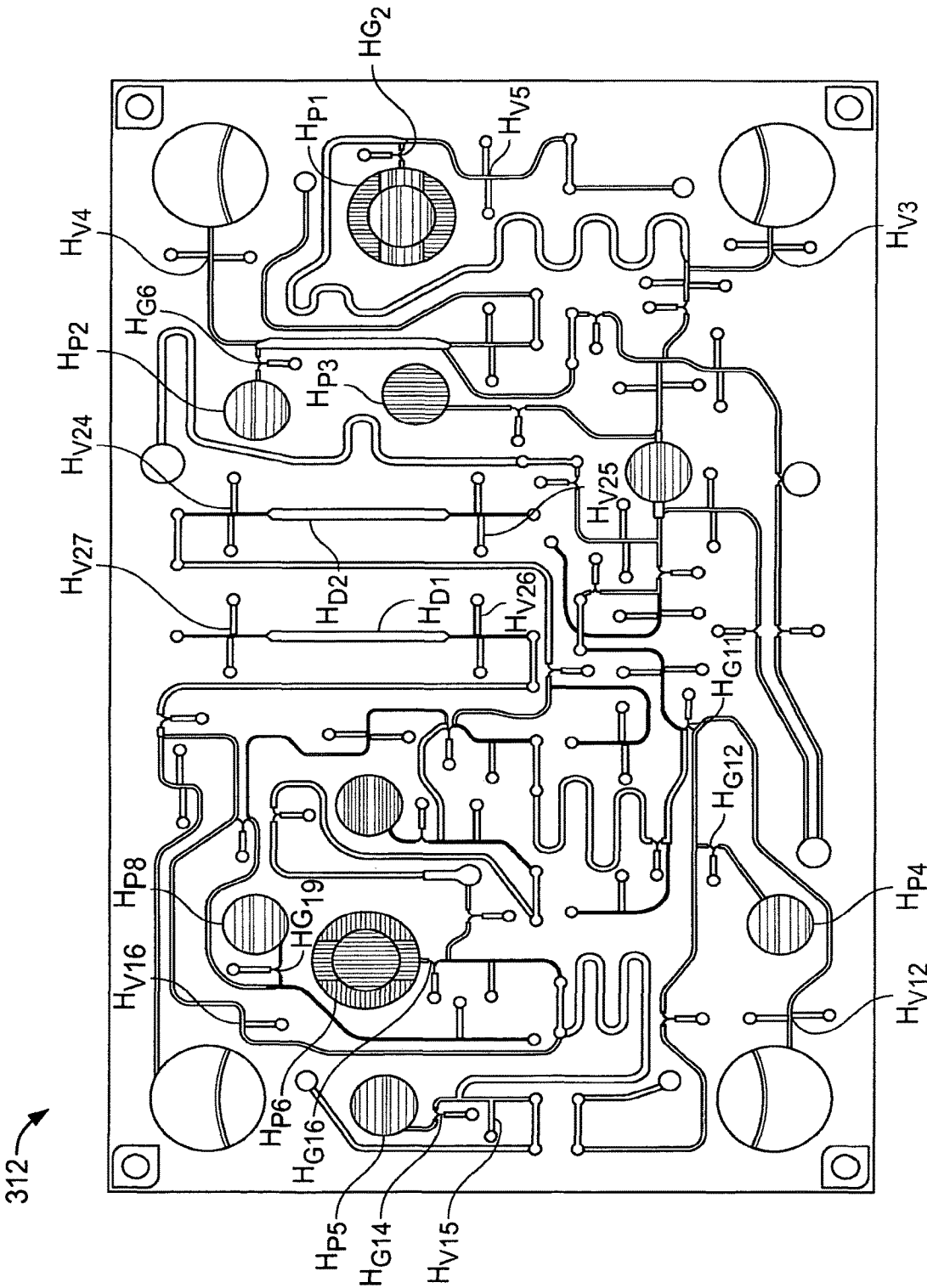


FIG. 7

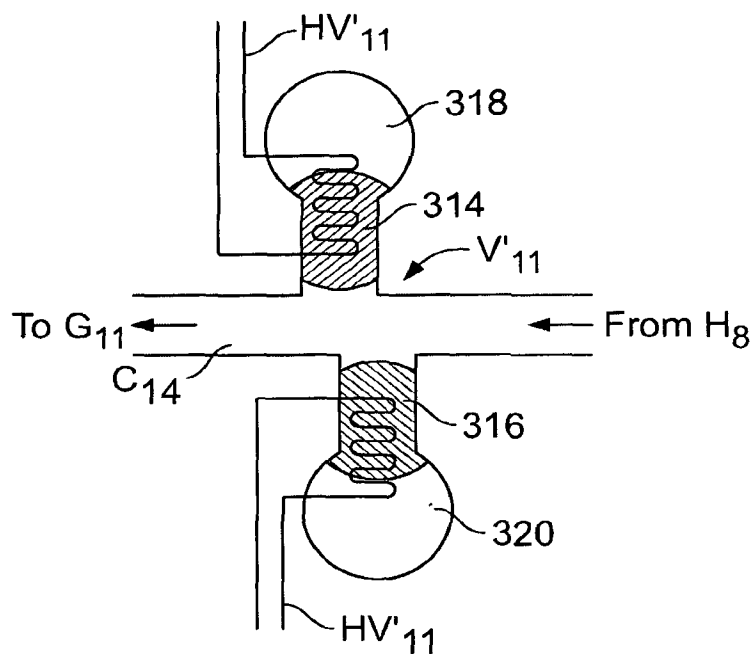


FIG. 8

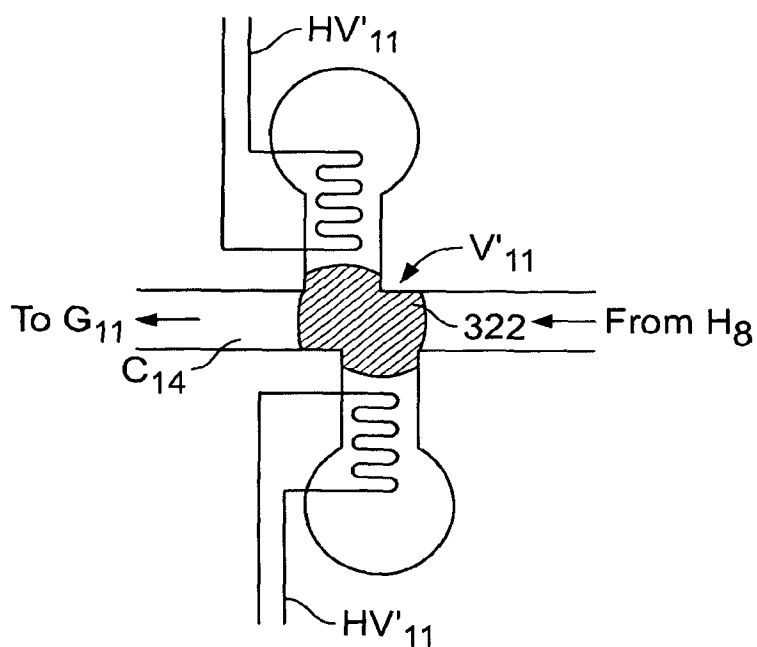


FIG. 9

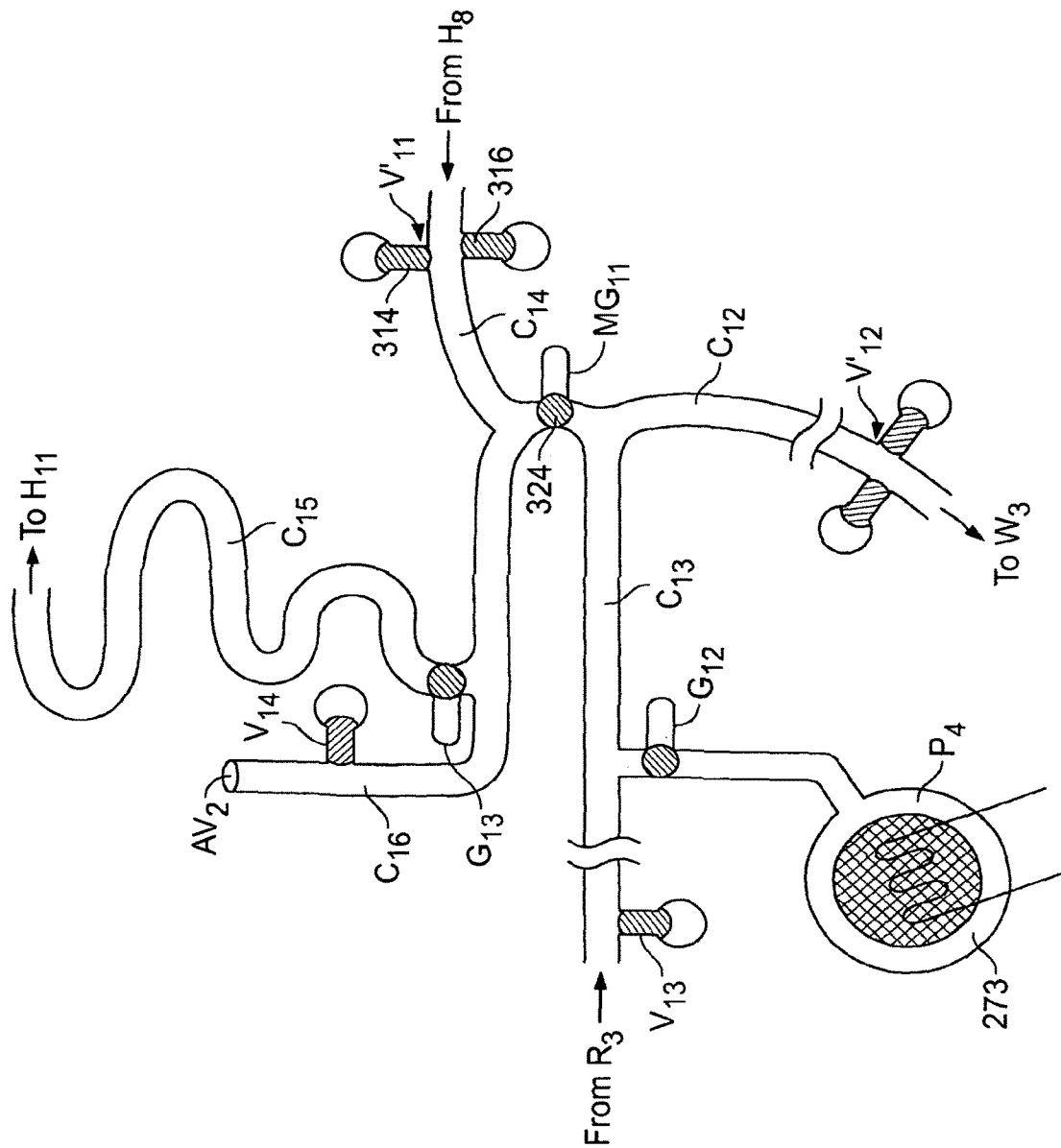


FIG. 10A

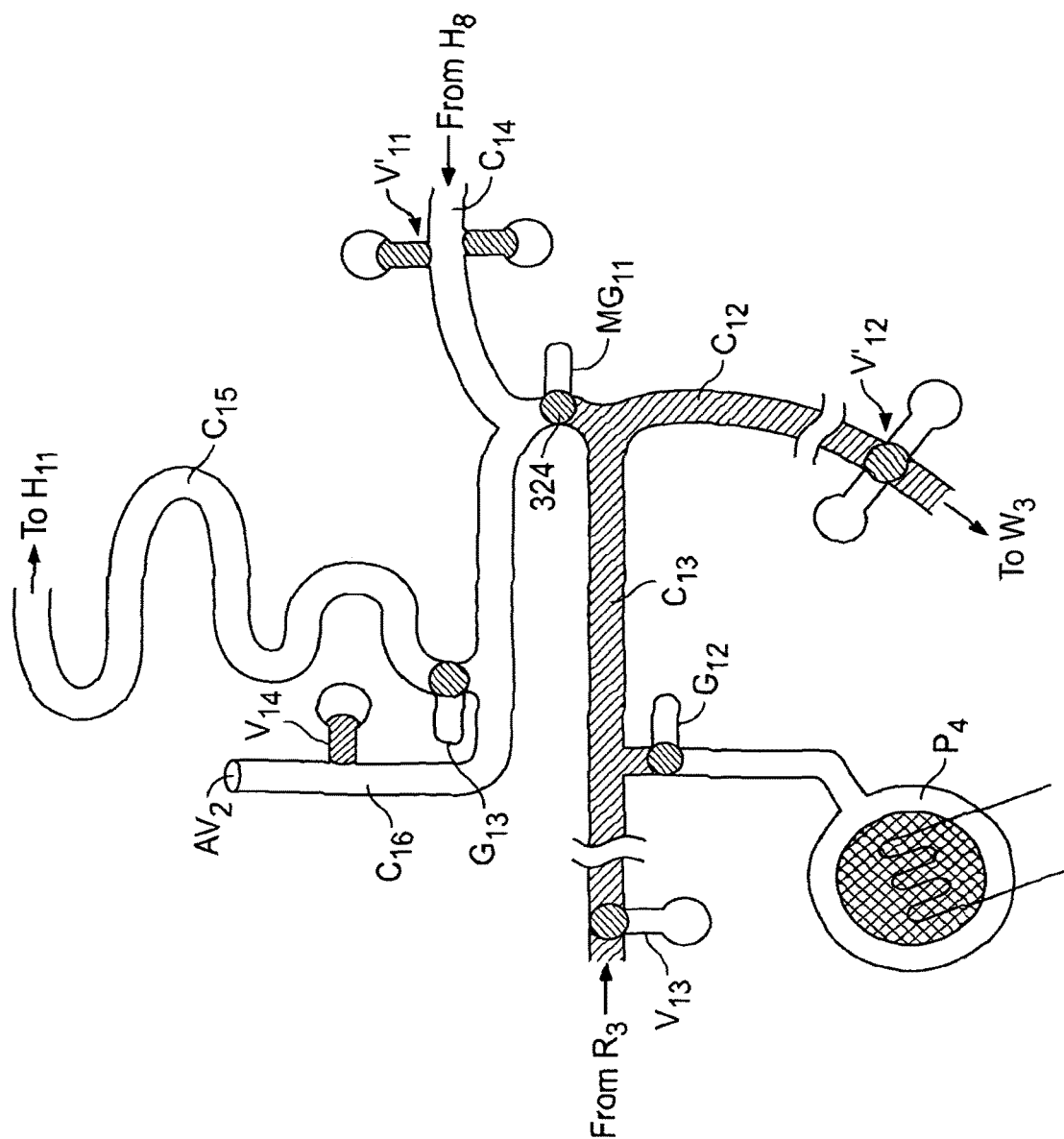


FIG. 10B

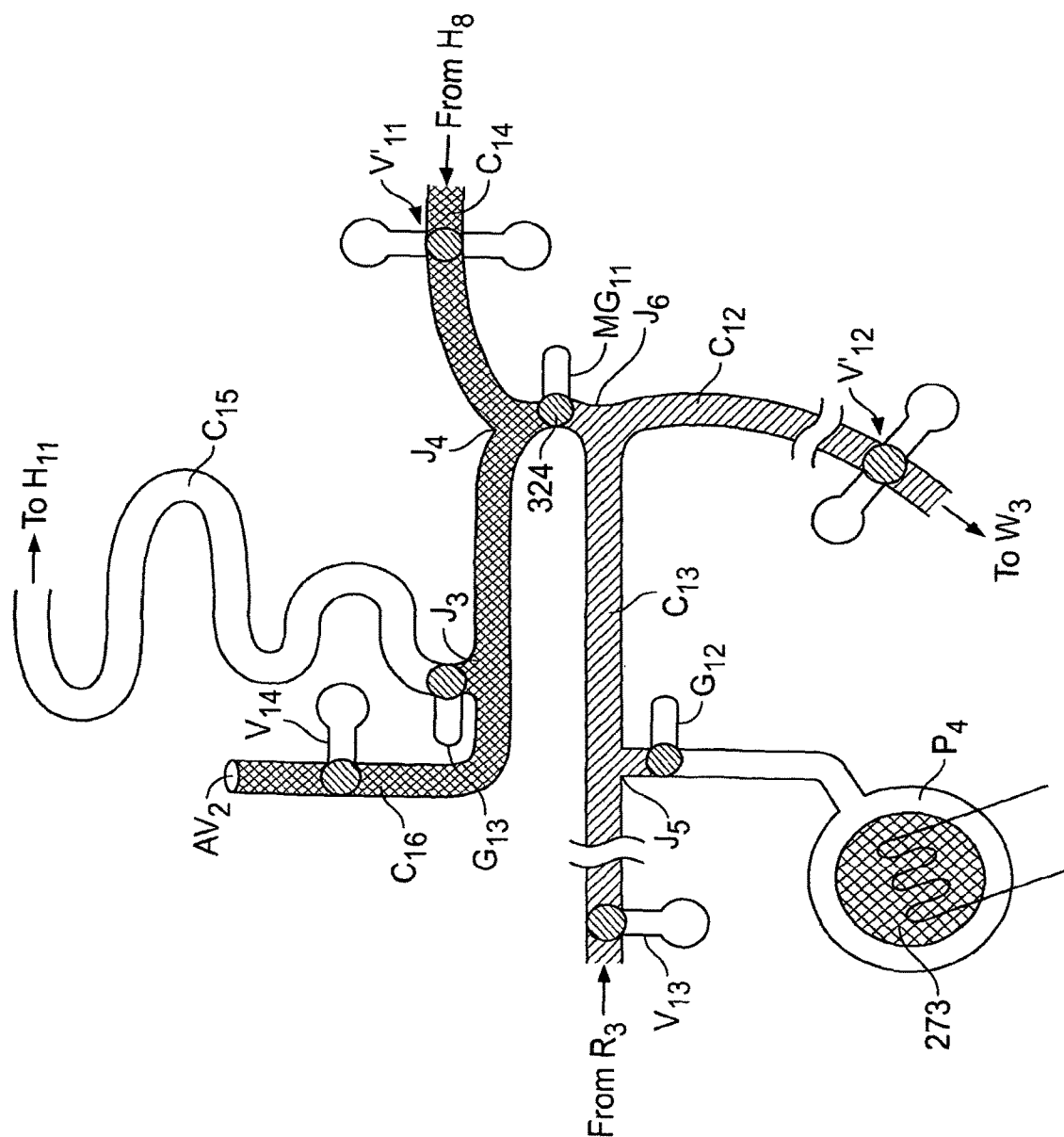


FIG. 10C

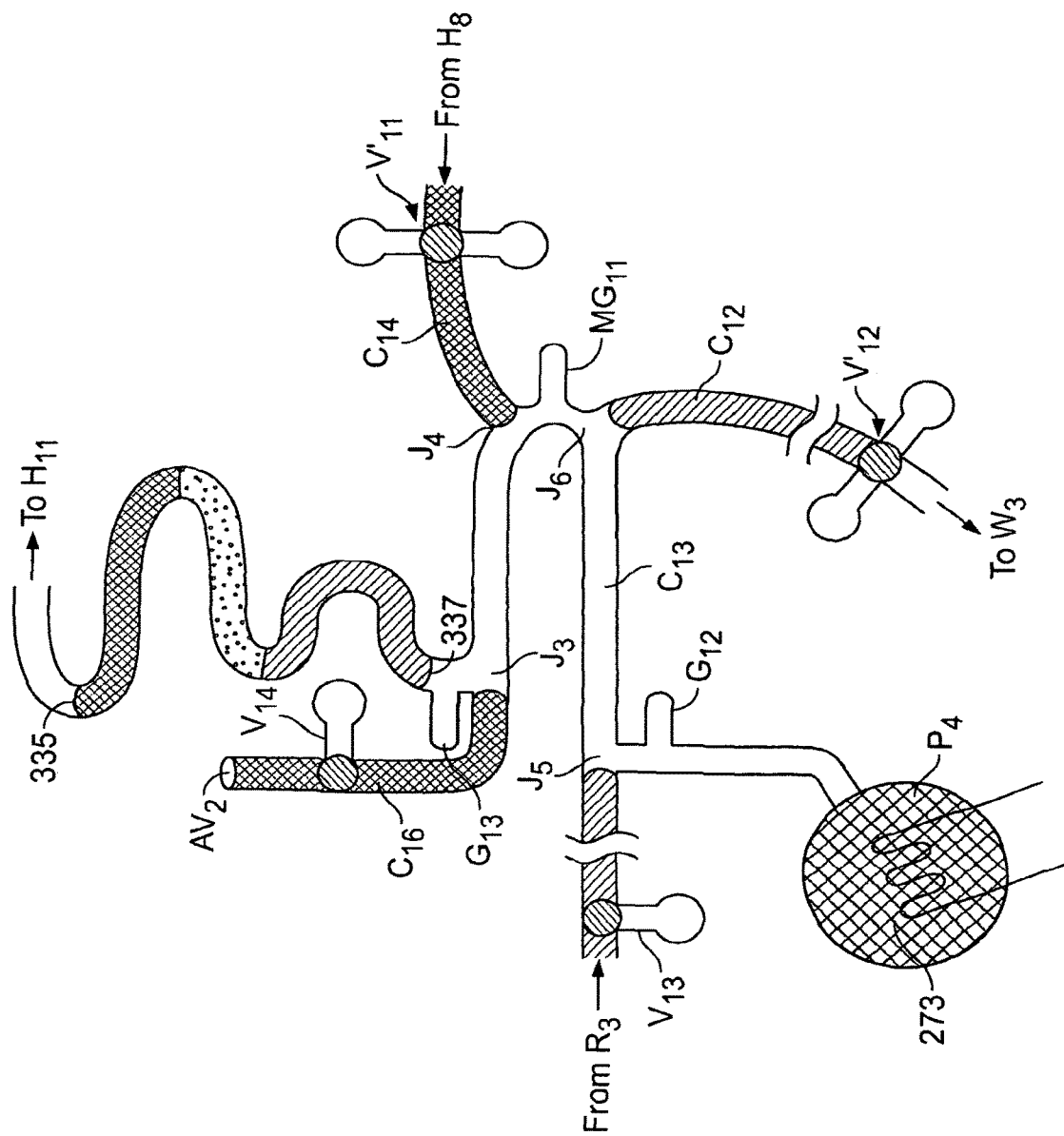


FIG. 10D

U.S. Patent

Dec. 3, 2019

Sheet 12 of 25

US 10,494,663 B1

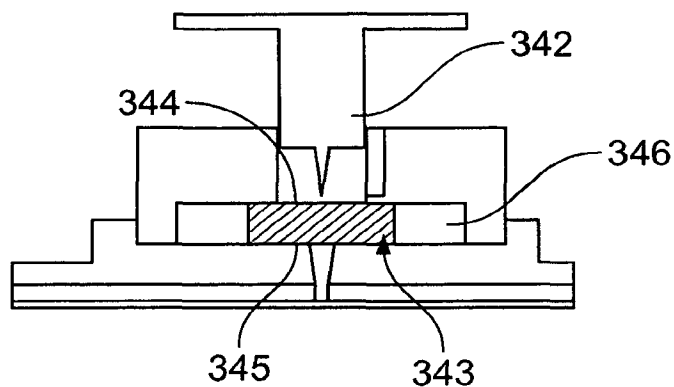


FIG. 11A

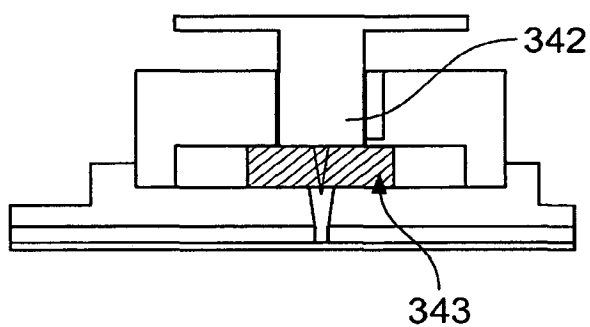


FIG. 11B

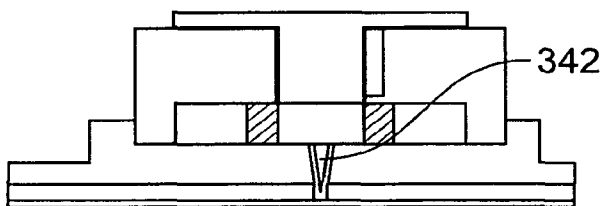


FIG. 11C

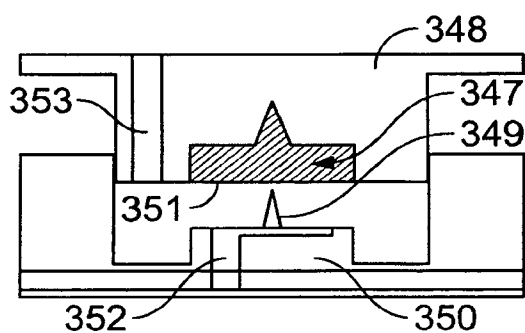


FIG. 12A

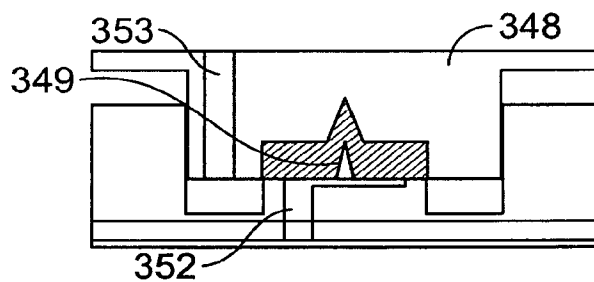


FIG. 12B

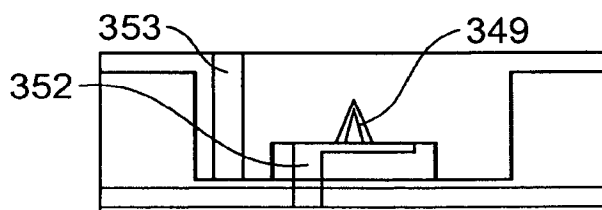


FIG. 12C

U.S. Patent

Dec. 3, 2019

Sheet 14 of 25

US 10,494,663 B1

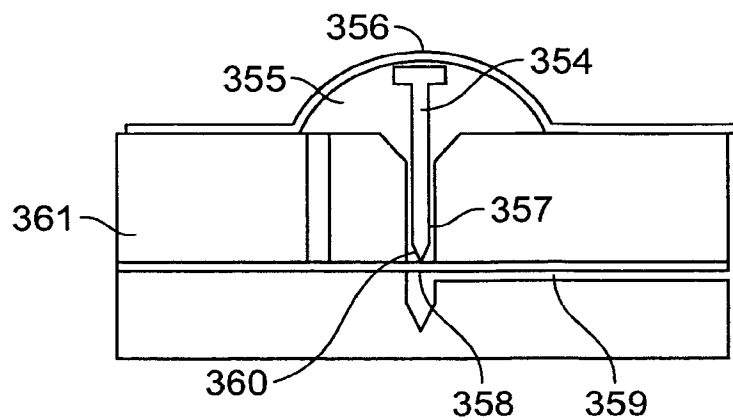


FIG. 13

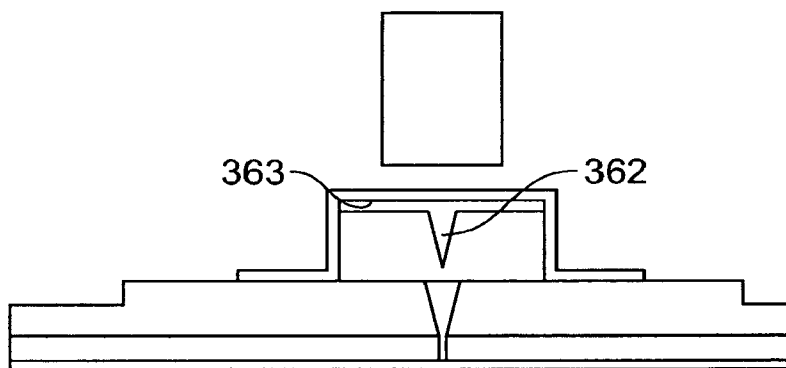


FIG. 14A

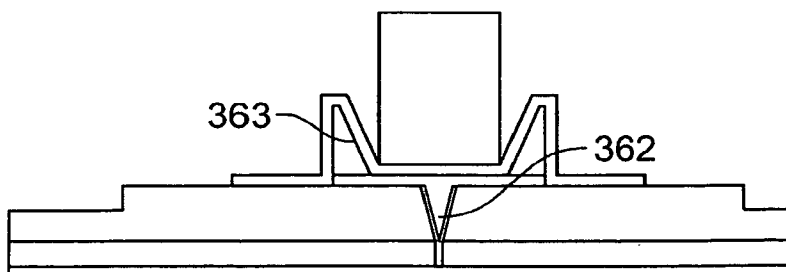


FIG. 14B

U.S. Patent

Dec. 3, 2019

Sheet 15 of 25

US 10,494,663 B1

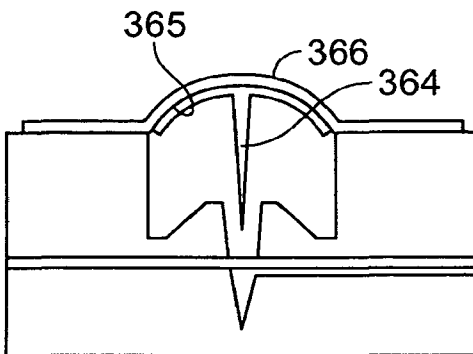


FIG. 15A

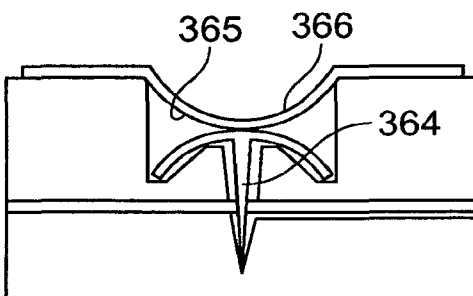


FIG. 15B

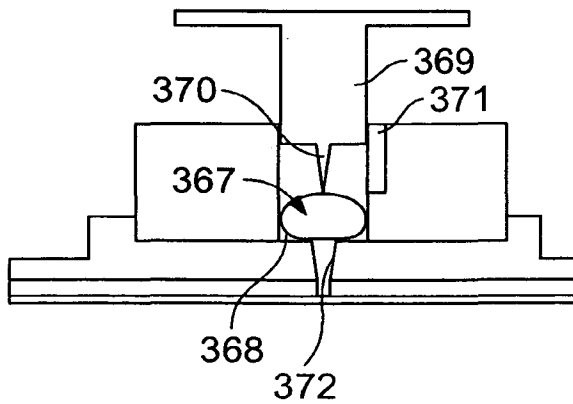


FIG. 16

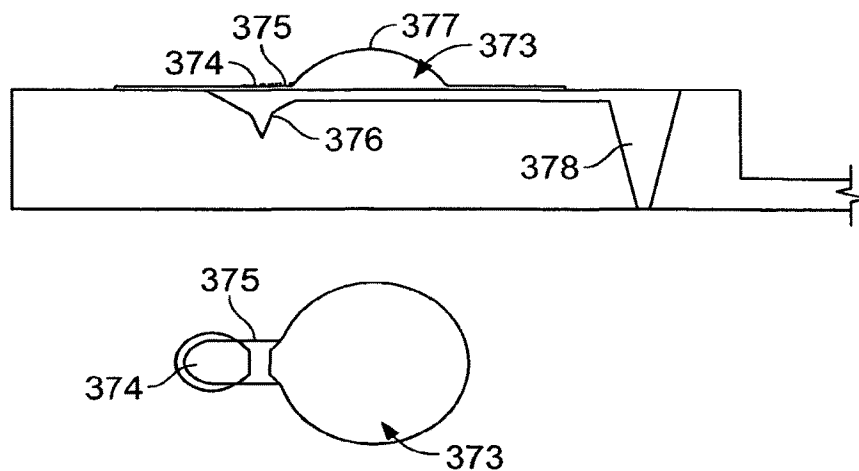


FIG. 17

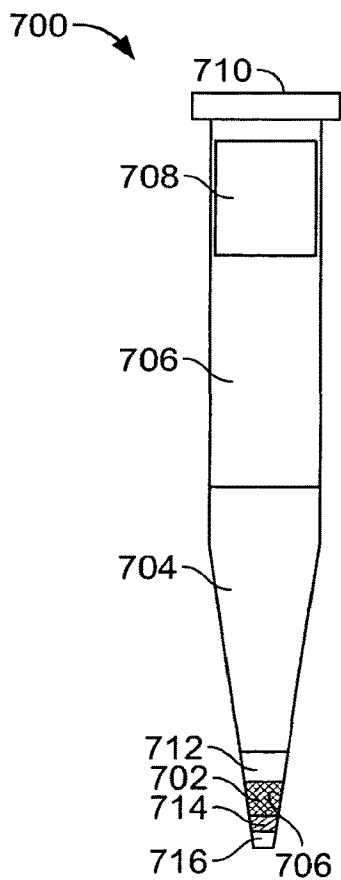


FIG. 18

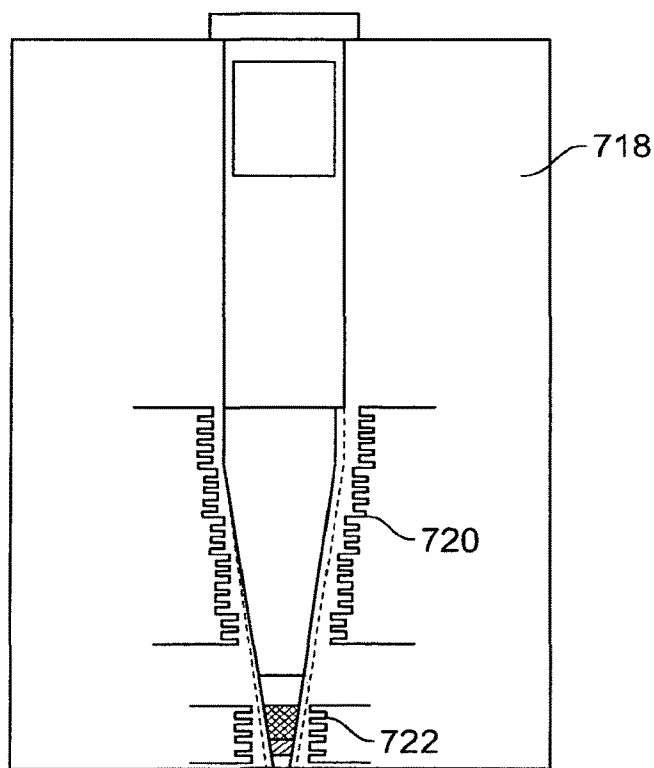


FIG. 19

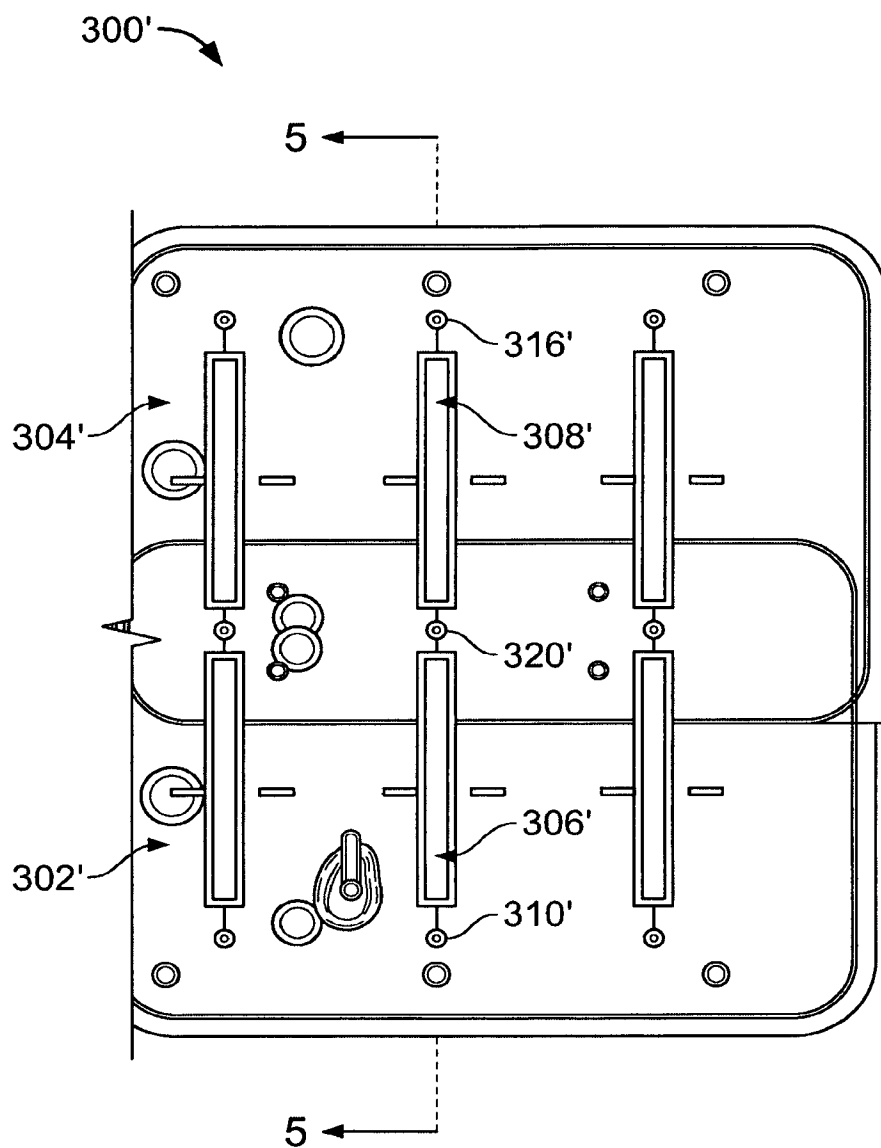


FIG. 20

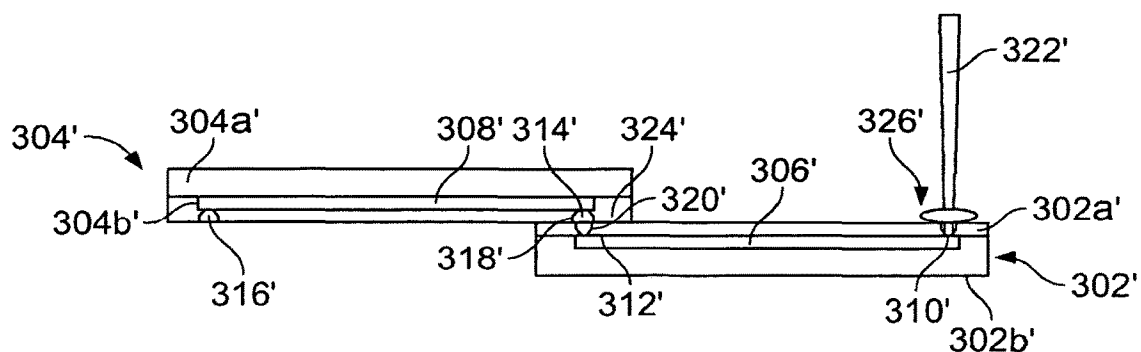


FIG. 21

DNA Capture by Poly-L-Lysine Beads

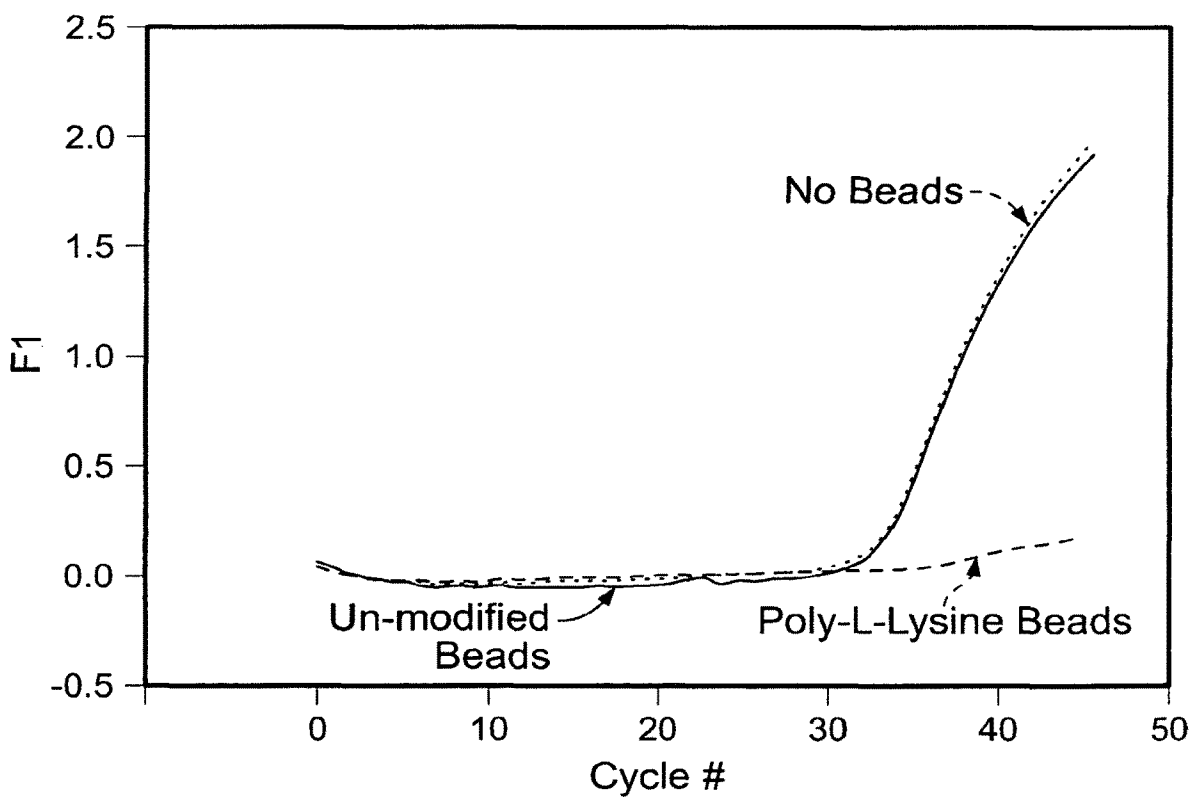


FIG. 22

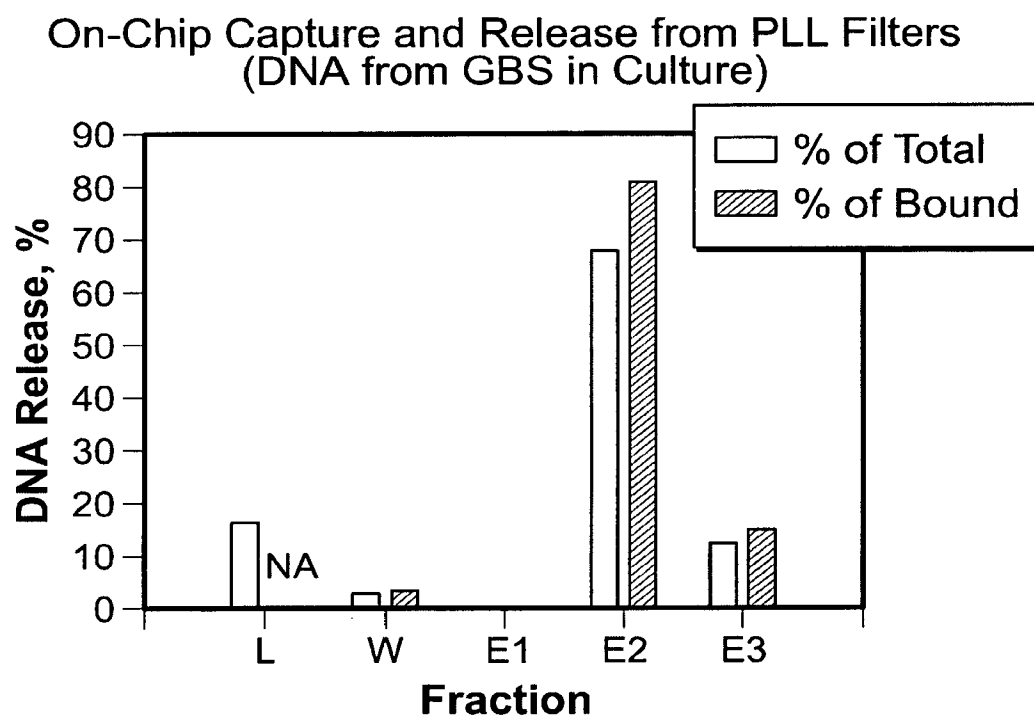


FIG. 23

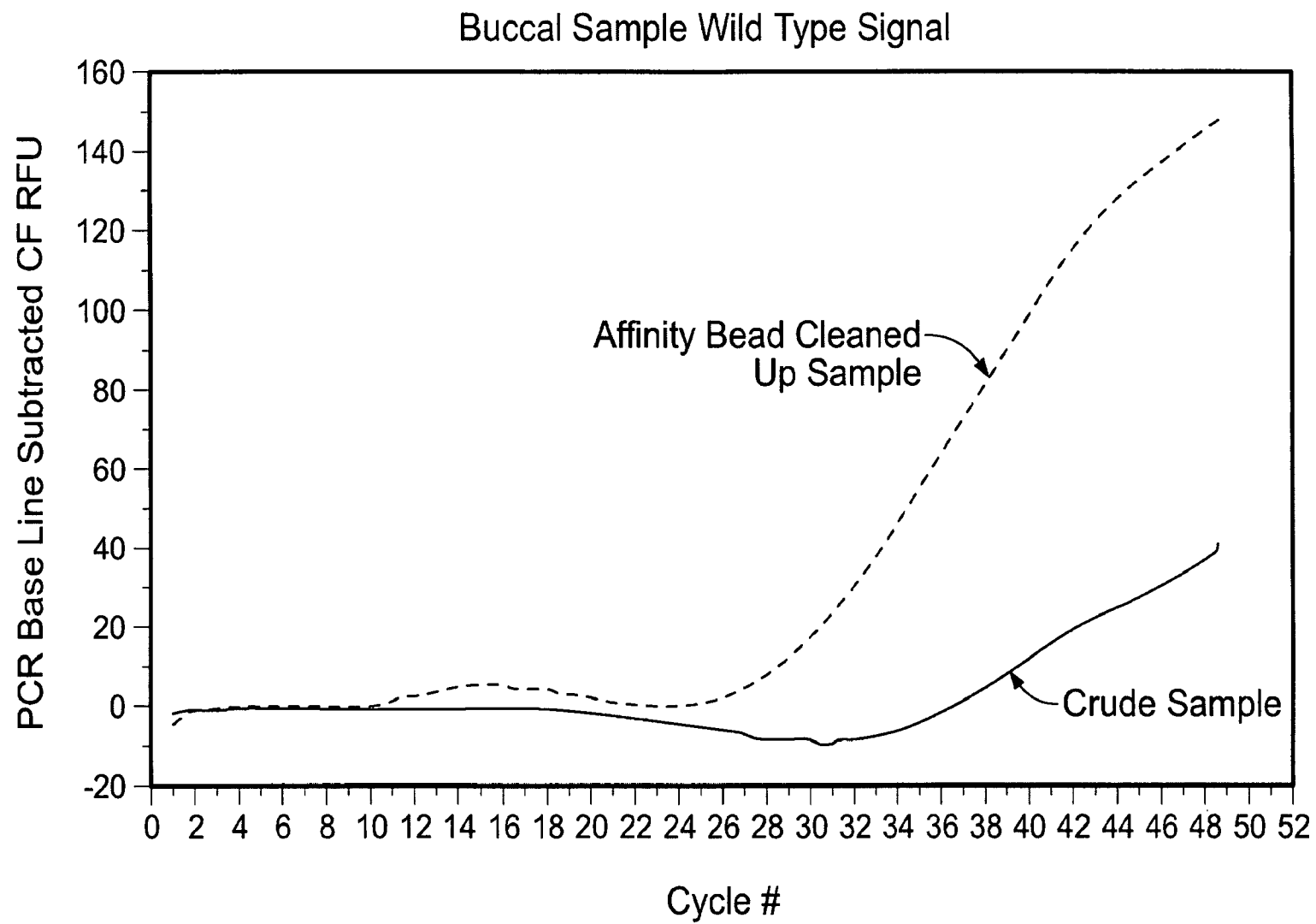


FIG. 24

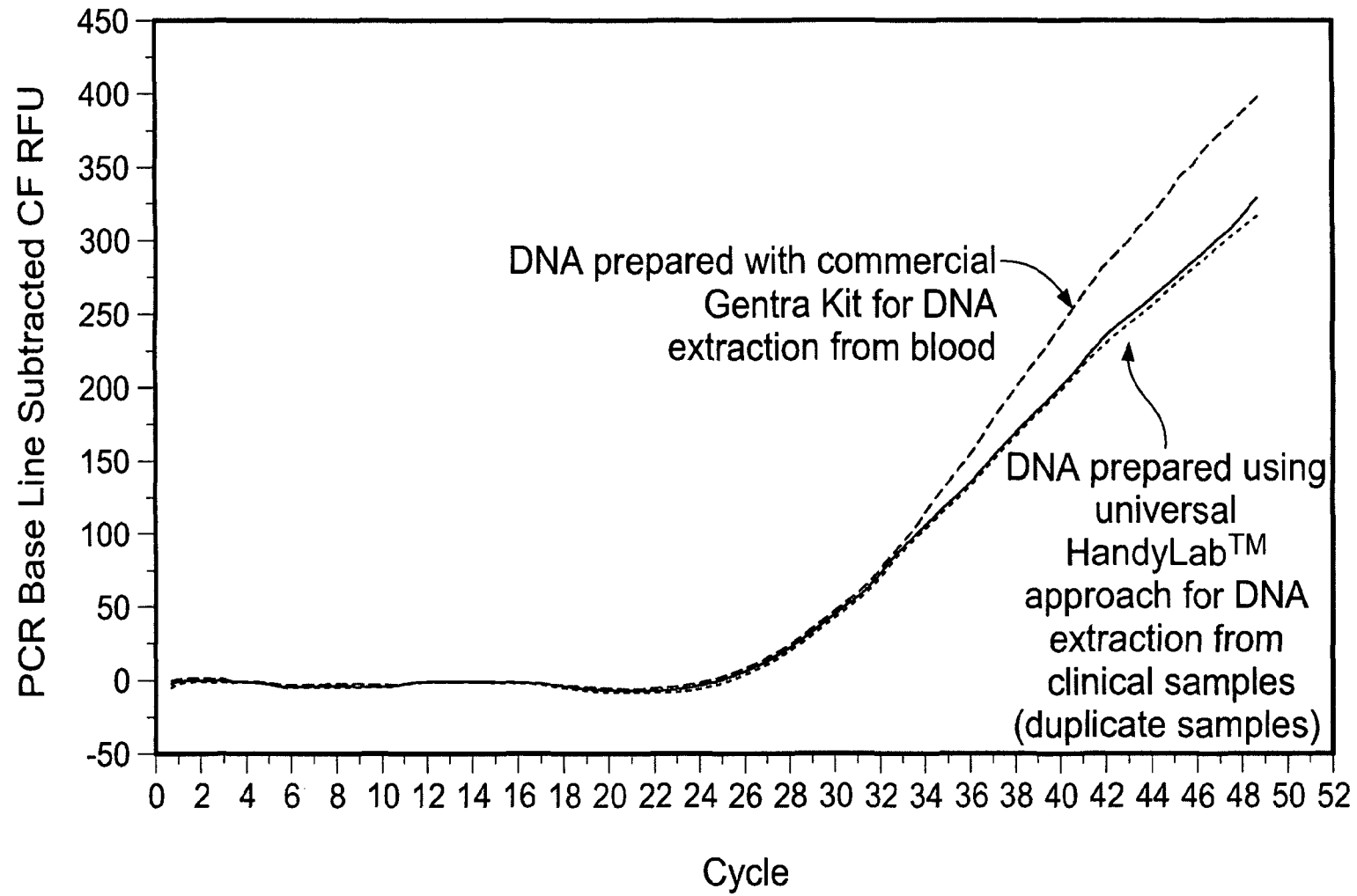


FIG. 25

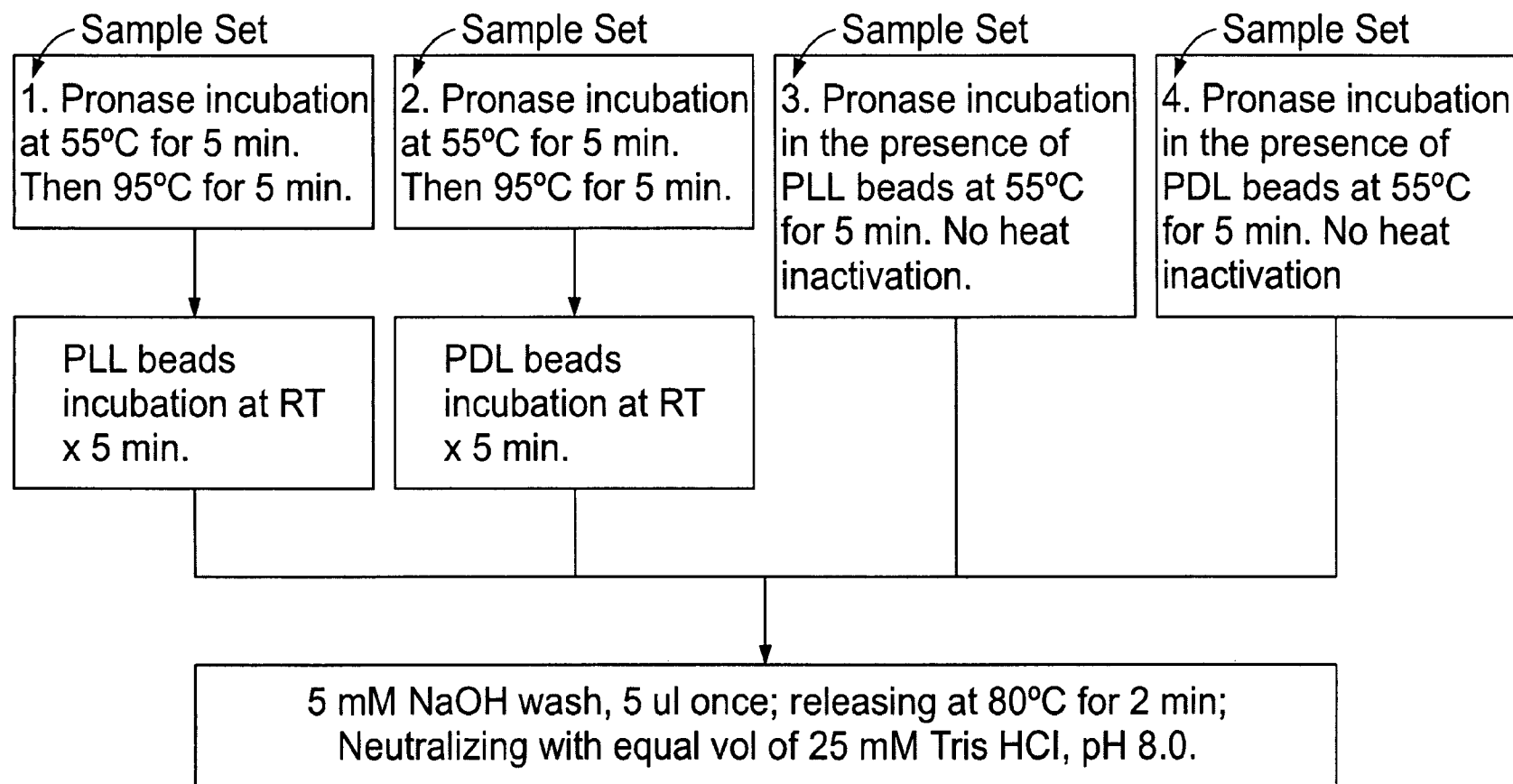


FIG. 26A

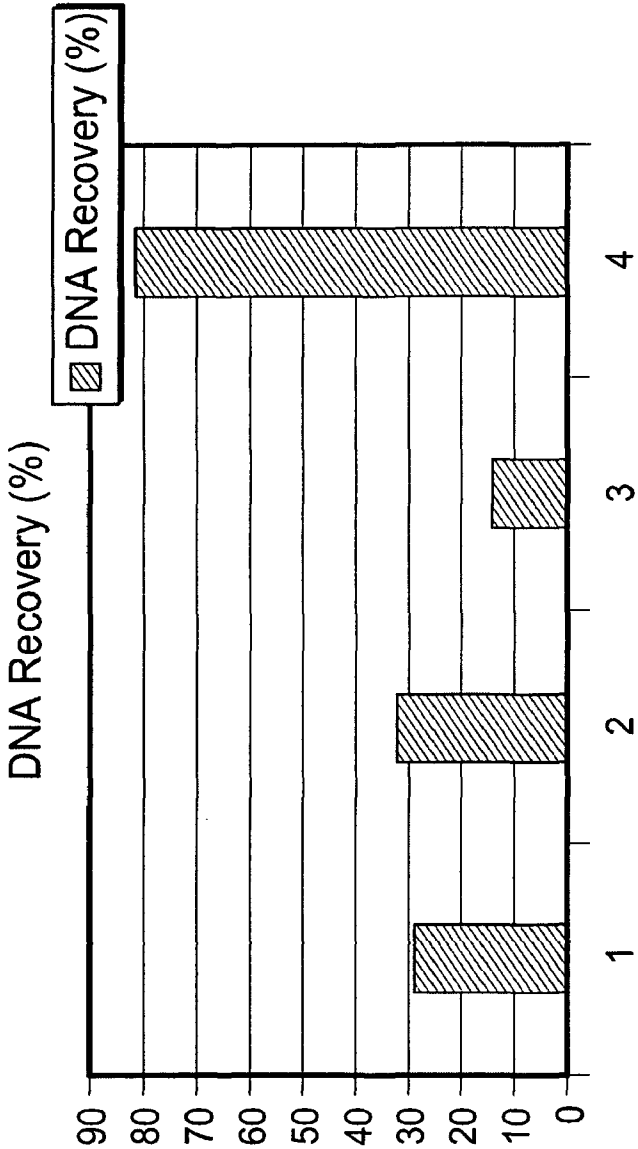


FIG. 26B

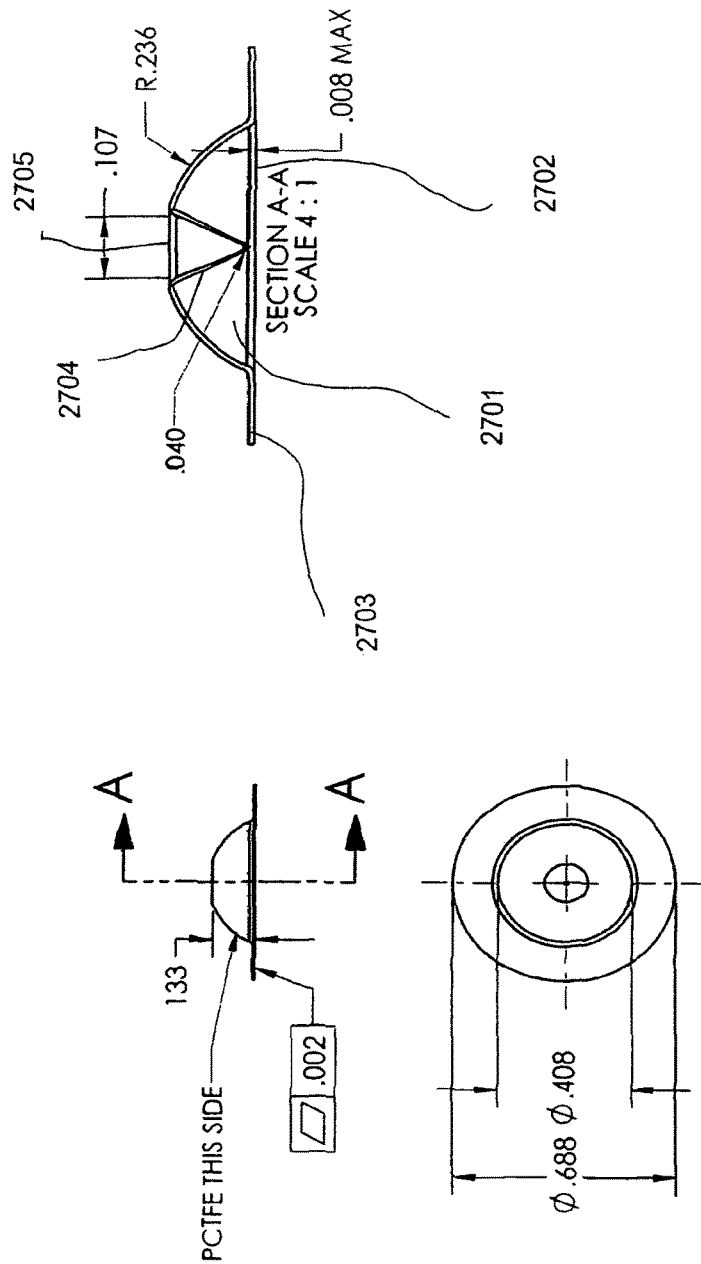


FIG. 27A

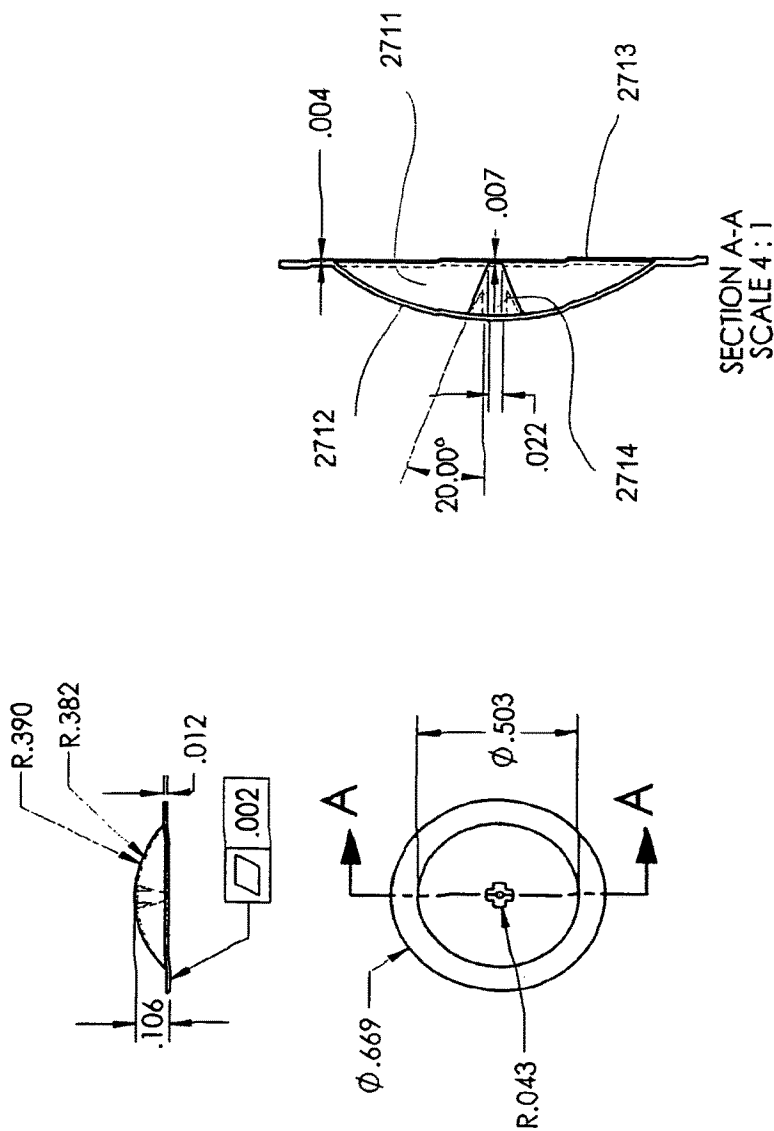


FIG. 27B

US 10,494,663 B1

1

METHOD FOR PROCESSING POLYNUCLEOTIDE-CONTAINING SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 16/518,735, filed Jul. 22, 2019, which is a continuation of U.S. patent application Ser. No. 14/506,471, filed Oct. 3, 2014 and issued as U.S. Pat. No. 10,364,456 on Jul. 30, 2019, which is a continuation of U.S. patent application Ser. No. 11/281,247, filed Nov. 16, 2005 and issued as U.S. Pat. No. 8,852,862 on Oct. 7, 2014, which is a continuation-in-part of International Application No. PCT/US2005/015345, filed May 3, 2005, which claims the benefit of priority of U.S. Provisional Application No. 60/567,174, filed May 3, 2004, and U.S. Provisional Application No. 60/645,784, filed Jan. 21, 2005. Each of these applications is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods for processing polynucleotide-containing samples as well as to related systems.

BACKGROUND

The analysis of a biological sample often includes detecting one or more polynucleotides present in the sample. One example of detection is qualitative detection, which relates, for example, to the determination of the presence of the polynucleotide and/or the determination of information related to, for example, the type, size, presence or absence of mutations, and/or the sequence of the polynucleotide. Another example of detection is quantitative detection, which relates, for example, to the determination of the amount of polynucleotide present. Detection may include both qualitative and quantitative aspects.

Detecting polynucleotides often involves the use of an enzyme. For example, some detection methods include polynucleotide amplification by polymerase chain reaction (PCR) or a related amplification technique. Other detection methods that do not amplify the polynucleotide to be detected also make use of enzymes. However, the functioning of enzymes used in such techniques may be inhibited by the presence of inhibitors present along with the polynucleotide to be detected. The inhibitors may interfere with, for example, the efficiency and/or specificity of the enzymes.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a method and related systems for processing one or more polynucleotides (e.g., to concentrate the polynucleotide(s) and/or to separate the polynucleotide(s) from inhibitor compounds (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucosal compounds, DNA binding proteins, or a saccharide) that might inhibit detection and/or amplification of the polynucleotides).

In some embodiments, the method includes contacting the polynucleotides and a relatively immobilized compound that preferentially associates with (e.g., retains) the polynucleotides as opposed to inhibitors. An exemplary compound is a poly-cationic polyamide (e.g., poly-L-lysine and/or poly-D-lysine), or polyethyleneimine (PEI), which may be bound to a surface (e.g., a surface of one or more particles). The

2

compound retains the polynucleotides so that the polynucleotides and inhibitors may be separated, such as by washing the surface with the compound and associated polynucleotides. Upon separation, the association between the polynucleotide and compound may be disrupted to release (e.g., separate) the polynucleotides from the compound and surface.

In some embodiments, the surface (e.g., a surface of one or more particles) is modified with a poly-cationic substance such as a polyamide or PEI, which may be covalently bound to the surface. The poly-cationic polyamide may include at least one of poly-L-lysine and poly-D-lysine. In some embodiments, the poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) have an average molecular weight of at least about 7500 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have an average molecular weight of less than about 35,000 Da (e.g., an average molecular weight of less than about 30000 Da (e.g., an average molecular weight of about 25,000 Da)). The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of at least about 15,000 Da. The poly-cationic polyamide (e.g., the at least one of the poly-L-lysine and the poly-D-lysine) may have a median molecular weight of less than about 25,000 Da (e.g., a median molecular weight of less than about 20,000 Da (e.g., a median molecular weight of about 20,000 Da)). If the polycationic material is PEI, its molecular weight is preferably in the range 600-800 Daltons.

Another aspect of the invention relates to a sample preparation device including a surface including a polycationic polyamide or PEI bound thereto and a sample introduction passage in communication with the surface for contacting the surface with a fluidic sample.

In some embodiments, the device includes a heat source configured to heat an aqueous liquid in contact with the surface to at least about 65° C.

In some embodiments, the device includes a reservoir of liquid having a pH of at least about 10 (e.g., about 10.5 or more). The device is configured to contact the surface with the liquid (e.g., by actuating a pressure source to move the liquid).

In some embodiments, the surface comprises surfaces of a plurality of particles.

In some embodiments, the poly-cationic polyamide includes poly-L-lysine and/or poly-D-lysine.

Another aspect of the invention relates to a method for processing a sample including providing a mixture including a liquid and an amount of polynucleotide, contacting a retention member with the mixture. The retention member may be configured to preferentially retain polynucleotides as compared to polymerase chain reaction inhibitors. Substantially all of the liquid in the mixture is removed from the retention member. The polynucleotides are released from the retention member. The polynucleotide may have a size of less than about 7.5 Mbp.

The liquid may be a first liquid and removing substantially all of the liquid from the retention member may include contacting the retention member with a second liquid.

Contacting the retention member with a second liquid can include actuating a thermally actuated pressure source to apply a pressure to the second liquid. Contacting the retention member with a second liquid can include opening a thermally actuated valve to place the second liquid in fluid communication with the retention member.

US 10,494,663 B1

3

The second liquid may have a volume of less than about 50 microliters.

The retention member may include a surface having a compound configured to bind polynucleotides preferentially to polymerase chain reaction inhibitors (e.g., hemoglobin, peptides, faecal compounds, humic acids, mucousol compounds, DNA binding proteins, or a saccharide).

The surface may include a poly-lysine (e.g., poly-L-lysine and/or poly-D-lysine) or PEI.

The second liquid may include a detergent (e.g., SDS).

Releasing may include heating the retention member to a temperature of at least about 50° C. (e.g., at about 65° C.). The temperature may be insufficient to boil the liquid in the presence of the retention member during heating. The temperature may be 100° C. or less (e.g., less than 100° C., about 97° C. or less). The temperature may be maintained for less than about 10 minutes (e.g., for less than about 5 minutes, for less than about 3 minutes).

The releasing may be performed without centrifugation of the retention member.

In certain embodiments, PCR inhibitors are rapidly removed from clinical samples to create a PCR-ready sample. The method may comprise the preparation of a polynucleotide-containing sample that is substantially free of inhibitors. The samples may be prepared from, e.g., crude lysates resulting from thermal, chemical, ultrasonic, mechanical, electrostatic, and other lysing techniques. The samples may be prepared without centrifugation. The samples may be prepared using microfluidic devices or on a larger scale.

Another aspect of the invention relates to a retention member, e.g., a plurality of particles such as beads, comprising bound PEI, or poly-lysine, e.g., poly-L-lysine, and related methods and systems. The retention member preferentially binds polynucleotides, e.g., DNA, as compared to inhibitors. The retention member may be used to prepare polynucleotides samples for further processing, such as amplification by polymerase chain reaction.

In certain embodiments, more than 90% of a polynucleotide present in a sample may be bound to the retention member, released, and recovered.

In certain embodiments, a polynucleotide may be bound to the retention member, released, and recovered, in less than about 10 minutes (e.g., less than about 7.5 minutes, less than about 5 minutes, or less than about 3 minutes).

A polynucleotide may be bound to a retention member, released, and recovered without subjecting the polynucleotide, retention member, and/or inhibitors to centrifugation. Separating the polynucleotides and inhibitors generally excludes subjecting the polynucleotides, inhibitors, processing region, and/or retention member to sedimentation (e.g., centrifugation).

Another aspect of the invention relates to a microfluidic device including a channel, a first mass of a thermally responsive substance (TRS) disposed on a first side of the channel, a second mass of a TRS disposed on a second side of the channel opposite the first side of the channel, a gas pressure source associated with the first mass of the TRS. Actuation of the gas pressure source drives the first mass of the TRS into the second mass of the TRS and obstructs the channel.

The microfluidic device can include a second gas pressure source associated with the second mass of the TRS. Actuation of the second gas pressure source drives the second mass of TRS into the first mass of TRS.

At least one (e.g., both) of the first and second masses of TRS may be a wax.

4

Another aspect of the invention relates to a method for obstructing a channel of a microfluidic device. A mass of a TRS is heated and driven across the channel (e.g., by gas pressure) into a second mass of TRS. The second mass of TRS may also be driven (e.g., by gas pressure) toward the first mass of TRS.

Another aspect of the invention relates to an actuator for a microfluidic device. The actuator includes a channel, a chamber connected to the channel, at least one reservoir of encapsulated liquid disposed in the chamber, and a gas surrounding the reservoir within the chamber. Heating the chamber expands the reservoir of encapsulated liquid and pressurizes the gas. Typically the liquid has a boiling point of about 90° C. or less. The liquid may be a hydrocarbon having about 10 carbon atoms or fewer.

The liquid may be encapsulated by a polymer.

The actuator may include multiple reservoirs of encapsulated liquid disposed in the chamber.

The multiple reservoirs may be dispersed within a solid (e.g., a wax).

The multiple reservoirs may be disposed within a flexible enclosure (e.g., a flexible sack).

Another aspect of the invention relates to a method including pressurizing a gas within a chamber of a microfluidic to create a gas pressure sufficient to move a liquid within a channel of the microfluidic device. Pressurizing the gas typically expanding at least one reservoir of encapsulated liquid disposed within the chamber.

Expanding the at least one reservoir can include heating the chamber.

Pressurizing the gas can include expanding multiple reservoirs of encapsulated liquid.

Another aspect of the invention relates to a method for combining (e.g., mixing) first and second liquids and related devices. The device includes a mass of a temperature responsive substance (TRS) that separates first and second channels of the device. The device is configured to move a first liquid along the first channel so that a portion (e.g., a medial portion) of the first liquid is adjacent the TRS and to move a second liquid along the second channel so that a portion (e.g., a medial portion) of second liquid is adjacent the TRS. A heat source is actuated to move the TRS (e.g., by melting, dispersing, fragmenting). The medial portions of the first and second liquids typically combine without being separated by a gas interface. Typically, only a subset of the first liquid and a subset of the second liquid are combined. The liquids mix upon being moved along a mixing channel.

Another aspect of the invention relates to a lyophilized reagent particle and a method of making the particle.

In some embodiments, the lyophilized particles include multiple smaller particles each having a plurality of ligands that preferentially associate with polynucleotides as compared to PCR inhibitors. The lyophilized particles can also (or alternatively) include lysing reagents (e.g., enzymes) configured to lyse cells to release polynucleotides. The lyophilized particles can also (or alternatively) include enzymes (e.g., proteases) that degrade proteins.

Cells can be lysed by combining a solution of the cells with the lyophilized particles to reconstitute the particles. The reconstituted lysing reagents lyse the cells. The polynucleotides associate with ligands of the smaller particles. During lysis, the solution may be heated (e.g., radiatively using a lamp (e.g., a heat lamp)).

In some embodiments, lyophilized particles include reagents (e.g., primers, control plasmids, polymerase enzymes) for performing PCR.

US 10,494,663 B1

5

A method for making lyophilized particles includes forming a solution of reagents of the particle and a cryoprotectant (e.g., a sugar or poly-alcohol). The solution is deposited dropwise on a chilled hydrophobic surface (e.g., a diamond film or polytetrafluoroethylene surface), without contacting a cooling agent such as liquid nitrogen. The particles freeze and are subjected to reduced pressure (typically while still frozen) for a time sufficient to remove (e.g., sublimate) the solvent. The lyophilized particles may have a diameter of about 5 mm or less (e.g., about 2.5 mm or less, about 1.75 mm or less).

Another aspect of the invention relates to a liquid reservoir capable of holding a liquid (e.g., a solvent, a buffer, a reagent, or combination thereof). In general, the reservoir can have one or more of the following features.

The reservoir can include a wall that can be manipulated (e.g., pressed or depressed) to decrease a volume within the reservoir. For example, the reservoir can include a piercing member (e.g., a needle-like or otherwise pointed or sharp member) that ruptures another portion of the reservoir (e.g., a portion of the wall) to release liquid. The piercing member can be internal to the reservoir such that the piercing member ruptures the wall from an inner surface of the reservoir (e.g., wall) outwards.

In general, the wall resists passage of liquid or vapor therethrough. In some embodiments, the wall lacks stretchiness. The wall may be flexible. The wall may be, e.g., a metallic layer, e.g., a foil layer, a polymer, or a laminate including a combination thereof.

The wall may be formed by vacuum formation (e.g., applying a vacuum and heat to a layer of material to draw the layer against a molding surface). The molding surface may be concave such that the wall is provided with a generally convex surface.

Exemplary liquids held by the reservoir include water and aqueous solutions including one or more salts (e.g., magnesium chloride, sodium chloride, Tris buffer, or combination thereof). The reservoir can retain the liquid (e.g., without substantial evaporation thereof) for a period of time (e.g., at least 6 months or at least a year). In some embodiments, less than 10% (e.g., less than about 5%) by weight of the liquid evaporates over a year.

The piercing member may be an integral part of a wall of the reservoir. For example, the reservoir can include a wall having an internal projection, which may be in contact with liquid in the reservoir. The reservoir also includes a second wall opposite the piercing member. During actuation, the piercing member is driven through the second wall (e.g., from the inside out) to release liquid.

In some embodiments, a maximum amount of liquid retained by a reservoir is less than about 1 ml. For example, a reservoir may hold about 500 microliters or less (e.g., 300 microliters or less). Generally, a reservoir holds at least about 25 microliters (e.g., at least about 50 microliters). The reservoir can introduce within about 10% of the intended amount of liquid (e.g., 50 ± 5 μ l).

The reservoir can deliver a predetermined amount of liquid that is substantially air-free (e.g., substantially gas-free). Upon introduction of the liquid, the substantially air and/or gas free liquid produces few or no bubbles large enough to obstruct movement of the liquid within the microfluidic device. Use of a piercing member internal to the reservoir can enhance an ability of the reservoir to deliver substantially air and/or gas free liquids.

In some embodiments, the reservoir can be actuated to release liquid by pressing (e.g., by one's finger or thumb or by mechanical pressure actuation). The pressure may be

6

applied directly to a wall of the reservoir or to a plunger having a piercing member. In embodiments, minimal pressure is required to actuate the reservoir. An automated system can be used to actuate (e.g., press upon) a plurality of reservoirs simultaneously or in sequence.

In some embodiments, the reservoir does not include a piercing member. Instead, internal pressure generated within the reservoir ruptures a wall of the reservoir allowing liquid to enter the microfluidic device.

Upon actuating a reservoir to introduce liquid into the microfluidic device, liquid generally does not withdraw back into the reservoir. For example, upon actuation, the volume of the reservoir may decrease to some minimum but generally does not increase so as to withdraw liquid back into the reservoir. For example, the reservoir may stay collapsed upon actuation. In such embodiments, the flexible wall may be flexible but lack hysteresis or stretchiness. Alternatively or in combination, the reservoir may draw in air from a vent without withdrawing any of the liquid.

Actuation of the reservoir may include driving a piercing member through a wall of the reservoir.

The reservoir preserves the reactivity and composition of reagents therein (e.g., the chemicals within the reservoir may exhibit little or no change in reactivity over 6 months or a year).

The flexible wall of the reservoir can limit or prevent leaching of chemicals therethrough. The reservoir can be assembled independently of a microfluidic device and then secured to the microfluidic device.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of a microfluidic device.

FIG. 2 is a cross-sectional view of a processing region for retaining polynucleotides and/or separating polynucleotides from inhibitors.

FIG. 3 is a cross-sectional view of an actuator.

FIG. 4 is a perspective view of a microfluidic device.

FIG. 5 is a side cross-sectional view of the microfluidic device of FIG. 4.

FIGS. 6A and 6B, taken together, illustrate a perspective view of a microfluidic network of the microfluidic device of FIG. 4.

FIG. 7 illustrates an array of heat sources for operating components of the microfluidic device of FIG. 4.

FIGS. 8 and 9 illustrate a valve in the open and closed states respectively.

FIG. 10A-10D illustrate a mixing gate of the microfluidic network of FIGS. 6A and 6B and adjacent regions of the network.

FIGS. 11A-11C illustrate a reservoir with actuation mechanism.

FIGS. 12A-12C illustrate a reservoir with actuation mechanism.

FIG. 13 illustrates a reservoir with actuation mechanism.

FIGS. 14A-14B illustrate a reservoir with actuation mechanism.

FIGS. 15A-15B illustrate a reservoir with actuation mechanism.

FIG. 16 illustrates a reservoir with actuation mechanism.

FIG. 17 illustrates a reservoir with actuation mechanism.

FIG. 18 illustrates a device for separating polynucleotides and inhibitors.

FIG. 19 illustrates the device of FIG. 18 and a device for operation thereof.

FIG. 20 illustrates a microfluidic device.

US 10,494,663 B1

7

FIG. 21 is a cross-section of the microfluidic device of FIG. 20 taken along 5.

FIG. 22 illustrates the retention of herring sperm DNA.

FIG. 23 illustrates the retention and release of DNA from group B streptococci;

FIG. 24 illustrates the PCR response of a sample from which inhibitors had been removed and of a sample from which inhibitors had not been removed.

FIG. 25 illustrates the PCR response of a sample prepared in accord with the invention and a sample prepared using a commercial DNA extraction method.

FIG. 26A illustrates a flow chart showing steps performed during a method for separating polynucleotides and inhibitors.

FIG. 26B illustrates DNA from samples subjected to the method of FIG. 26A.

FIGS. 27A and 27B show, respectively, two embodiments of a reservoir with a piercing member.

DETAILED DESCRIPTION OF THE INVENTION

Analysis of biological samples often includes determining whether one or more polynucleotides (e.g., a DNA, RNA, mRNA, or rRNA) is present in the sample. For example, one may analyze a sample to determine whether a polynucleotide indicative of the presence of a particular pathogen is present. Typically, biological samples are complex mixtures. For example, a sample may be provided as a blood sample, a tissue sample (e.g., a swab of, for example, nasal, buccal, anal, or vaginal tissue), a biopsy aspirate, a lysate, as fungi, or as bacteria. Polynucleotides to be determined may be contained within particles (e.g., cells (e.g., white blood cells and/or red blood cells), tissue fragments, bacteria (e.g., gram positive bacteria and/or gram negative bacteria), fungi, spores). One or more liquids (e.g., water, a buffer, blood, blood plasma, saliva, urine, spinal fluid, or organic solvent) is typically part of the sample and/or is added to the sample during a processing step.

Methods for analyzing biological samples include providing a biological sample (e.g., a swab), releasing polynucleotides from particles (e.g., bacteria) of the sample, amplifying one or more of the released polynucleotides (e.g., by polymerase chain reaction (PCR)), and determining the presence (or absence) of the amplified polynucleotide(s) (e.g., by fluorescence detection). Biological samples, however, typically include inhibitors (e.g., mucosal compounds, hemoglobin, faecal compounds, and DNA binding proteins) that can inhibit determining the presence of polynucleotides in the sample. For example, such inhibitors can reduce the amplification efficiency of polynucleotides by PCR and other enzymatic techniques for determining the presence of polynucleotides. If the concentration of inhibitors is not reduced relative to the polynucleotides to be determined, the analysis can produce false negative results.

We describe methods and related systems for processing biological samples (e.g., samples having one or more polynucleotides to be determined). Typically, the methods and systems reduce the concentration of inhibitors relative to the concentration of polynucleotides to be determined.

Referring to FIG. 1, a microfluidic device 200 includes first, second, and third layers 205, 207, and 209 that define a microfluidic network 201 having various components configured to process a sample including one or more polynucleotides to be determined. Device 200 typically processes the sample by increasing the concentration of a polynucleotide to be determined and/or by reducing the

8

concentration of inhibitors relative to the concentration of polynucleotide to be determined.

We now discuss the arrangement of components of network 201.

Network 201 includes an inlet 202 by which sample material can be introduced to the network and an output 236 by which a processed sample can be removed (e.g., expelled by or extracted from) network 201. A channel 204 extends between inlet 202 and a junction 255. A valve 206 is positioned along channel 204. A reservoir channel 240 extends between junction 255 and an actuator 244. Gates 242 and 246 are positioned along channel 240. A channel 257 extends between junction 255 and a junction 259. A valve 208 is positioned along channel 257. A reservoir channel 246 extends between junction 259 and an actuator 248. Gates 250 and 252 are positioned along channel 246. A channel 261 extends between junction 259 and a junction 263. A valve 210 and a hydrophobic vent 212 are positioned along channel 261. A channel 256 extends between junction 263 and an actuator 254. A gate 258 is positioned along channel 256.

A channel 214 extends between junction 263 and a processing chamber 220, which has an inlet 265 and an outlet 267. A channel 228 extends between processing chamber outlet 267 and a waste reservoir 232. A valve 234 is positioned along channel 228. A channel 230 extends between processing chamber outlet 267 and output 236.

We turn now to particular components of network 201.

Referring also to FIG. 2, processing chamber 220 includes a plurality of particles (e.g., beads, microspheres) 218 configured to retain polynucleotides of the sample under a first set of conditions (e.g., a first temperature and/or first pH) and to release the polynucleotides under a second set of conditions (e.g., a second, higher temperature and/or a second, more basic pH). Typically, the polynucleotides are retained preferentially as compared to inhibitors that may be present in the sample. Particles 218 are configured as a retention member 216 (e.g., a column) through which sample material (e.g., polynucleotides) must pass when moving between the inlet 265 and outlet 267 of processing region 220.

A filter 219 prevents particles 218 from passing downstream of processing region 220. A channel 287 connects filter 219 with outlet 267. Filter 219 has a surface area within processing region 220 that is larger than the cross-sectional area of inlet 265. For example, in some embodiments, the ratio of the surface area of filter 219 within processing region 220 to the cross-sectional area of inlet 265 (which cross-sectional area is typically about the same as the cross-sectional area of channel 214) is at least about 5 (e.g., at least about 10, at least about 20, at least about 30). In some embodiments, the surface area of filter 219 within processing region 220 is at least about 1 mm² (e.g., at least about 2 mm², at least about 3 mm²). In some embodiments, the cross-sectional area of inlet 265 and/or channel 214 is about 0.25 mm² or less (e.g., about 0.2 mm² or less, about 0.15 mm² or less, about 0.1 mm² or less). The larger surface area presented by filter 219 to material flowing through processing region 220 helps prevent clogging of the processing region while avoiding significant increases in the void volume (discussed below) of the processing region.

Particles 218 are modified with at least one ligand that retains polynucleotides (e.g., preferentially as compared to inhibitors). Typically, the ligands retain polynucleotides from liquids having a pH about 9.5 or less (e.g., about 9.0 or less, about 8.75 or less, about 8.5 or less). As a sample solution moves through processing region 220, polynucle-

US 10,494,663 B1

9

otides are retained while the liquid and other solution components (e.g., inhibitors) are less retained (e.g., not retained) and exit the processing region. In general, the ligands release polynucleotides when the pH is about 10 or greater (e.g., about 10.5 or greater, about 11.0 or greater, about 11.4 or greater). Consequently, polynucleotides can be released from the ligand modified particles into the surrounding liquid.

Exemplary ligands include, for example, polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine) and PEI. Other ligands include, for example, intercalators, poly-intercalators, minor groove binders polyamines (e.g., spermidine), homopolymers and copolymers comprising a plurality of amino acids, and combinations thereof. In some embodiments, the ligands have an average molecular weight of at least about 5000 Da (e.g., at least about 7500 Da, of at least about 15000 Da). In some embodiments, the ligands have an average molecular weight of about 50000 Da or less (e.g., about 35000, or less, about 27500 Da or less). In some embodiments, the ligand is a poly-lysine ligand attached to the particle surface by an amide bond.

In certain embodiments, the ligands are resistant to enzymatic degradation, such as degradation by protease enzymes (e.g., mixtures of endo- and exo-proteases such as pronase) that cleave peptide bonds. Exemplary protease resistant ligands include, for example, poly-D-lysine and other ligands that are enantiomers of ligands susceptible to enzymatic attack.

Particles **218** are typically formed of a material to which the ligands can be associated. Exemplary materials from which particles **218** can be formed include polymeric materials that can be modified to attach a ligand. Typical polymeric materials provide or can be modified to provide carboxylic groups and/or amino groups available to attach ligands. Exemplary polymeric materials include, for example, polystyrene, latex polymers (e.g., polycarboxylate coated latex), polyacrylamide, polyethylene oxide, and derivatives thereof. Polymeric materials that can be used to form particles **218** are described in U.S. Pat. No. 6,235,313 to Mathiowitz et al., which patent is incorporated herein by reference. Other materials include glass, silica, agarose, and amino-propyl-tri-ethoxy-silane (APES) modified materials.

Exemplary particles that can be modified with suitable ligands include carboxylate particles (e.g., carboxylate modified magnetic beads (Sera-Mag Magnetic Carboxylate modified beads, Part #3008050250, Seradyn) and Polybead carboxylate modified microspheres available from Polyscience, catalog no. 09850). In some embodiments, the ligands include poly-D-lysine and the beads comprise a polymer (e.g., polycarboxylate coated latex). In other embodiments, the ligands include PEI.

In general, the ratio of mass of particles to the mass of polynucleotides retained by the particles is no more than about 25 or more (e.g., no more than about 20, no more than about 10). For example, in some embodiments, about 1 gram of particles retains about 100 milligrams of polynucleotides.

Typically, the total volume of processing region **220** (including particles **218**) between inlet **265** and filter **219** is about 15 microliters or less (e.g., about 10 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. In some embodiments, particles **218** occupy at least about 10 percent (e.g., at least about 15 percent) of the total volume of processing region **220**. In some embodiments, particles **218** occupy about 75 percent or less (e.g.,

10

about 50 percent or less, about 35 percent or less) of the total volume of processing chamber **220**.

In some embodiments, the volume of processing region **220** that is free to be occupied by liquid (e.g., the void volume of processing region **220** including interstices between particles **218**) is about equal to the total volume minus the volume occupied by the particles. Typically, the void volume of processing region **220** is about 10 microliters or less (e.g., about 7.5 microliters or less, about 5 microliters or less, about 2.5 microliters or less, about 2 microliters or less). In some embodiments, the void volume is about 50 nanoliters or more (e.g., about 100 nanoliters or more, about 250 nanoliters or more). In an exemplary embodiment, the total volume of processing region **220** is about 2.3 microliters. For example, in an exemplary embodiment, the total volume of the processing region is about 2.3 microliters, the volume occupied by particles is about 0.3 microliters, and the volume free to be occupied by liquid (void volume) is about 2 microliters.

Particles **218** typically have an average diameter of about 20 microns or less (e.g., about 15 microns or less, about 10 microns or less). In some embodiments, particles **218** have an average diameter of at least about 4 microns (e.g., at least about 6 microns, at least about 8 microns).

In some embodiments, a volume of channel **287** between filter **219** and outlet **267** is substantially smaller than the void volume of processing region **220**. For example, in some embodiments, the volume of channel **287** between filter **219** and outlet **267** is about 35% or less (e.g., about 25% or less, about 20% or less) of the void volume. In an exemplary embodiment, the volume of channel **287** between filter **219** and outlet **267** is about 500 nanoliters.

The particle density is typically at least about 10^8 particles per milliliter (e.g., about 10^9 particles per milliliter). For example, a processing region with a total volume of about 1 microliter may include about 103 beads.

Filter **219** typically has pores with a width smaller than the diameter of particles **218**. In an exemplary embodiment, filter **219** has pores having an average width of about 8 microns and particles **218** have an average diameter of about 10 microns.

In some embodiments, at least some (e.g., all) of the particles are magnetic. In alternative embodiments, few (e.g., none) of the particles are magnetic.

In some embodiments, at least some (e.g., all) the particles are solid. In some embodiments, at least some (e.g., all) the particles are porous (e.g., the particles may have channels extending at least partially within them).

We continue discussing components of network **201**.

Channels of microfluidic network **201** typically have at least one sub-millimeter cross-sectional dimension. For example, channels of network **201** may have a width and/or a depth of about 1 mm or less (e.g., about 750 microns or less, about 500 microns, or less, about 250 microns or less).

A valve is a component that has a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Upon actuation, the valve transitions to a closed state that prevents material from passing along the channel from one side of the valve to the other. For example, valve **206** includes a mass **251** of a thermally responsive substance (TRS) that is relatively immobile at a first temperature and more mobile at a second temperature. A chamber **253** is in gaseous communication with mass **251**. Upon heating gas (e.g., air) in chamber **253** and heating mass **251** of TRS to the second temperature, gas pressure within chamber **253**

US 10,494,663 B1

11

moves mass **251** into channel **204** obstructing material from passing therealong. Other valves of network **201** have the same structure and operate in the same fashion as valve **206**.

A mass of TRS can be an essentially solid mass or an agglomeration of smaller particles that cooperate to obstruct the passage. Examples of TRS's include a eutectic alloy (e.g., a solder), wax (e.g., an olefin), polymers, plastics, and combinations thereof. The first and second temperatures are insufficiently high to damage materials, such as polymer layers of device **200**. Generally, the second temperature is less than about 90° C. and the first temperature is less than the second temperature (e.g., about 70° C. or less).

A gate is a component that has a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Upon actuation, the gate transitions to an open state in which material is permitted to pass from one side of the gate (e.g., upstream of the gate) to the other side of the gate (e.g., downstream of the gate). For example, gate **242** includes a mass **271** of TRS positioned to obstruct passage of material between junction **255** and channel **240**. Upon heating mass **271** to the second temperature, the mass changes state (e.g., by melting, by dispersing, by fragmenting, and/or dissolving) to permit passage of material between junction **255** and channel **240**.

The portion of channel **240** between gates **242** and **246** forms a fluid reservoir **279** configured to hold a liquid (e.g., water, an organic liquid, or combination thereof). During storage, gates **242** and **246** limit (e.g., prevent) evaporation of liquid within the fluid reservoir. During operation of device **200**, the liquid of reservoir **279** is typically used as a wash liquid to remove inhibitors from processing region **220** while leaving polynucleotides associated with particles **218**. Typically, the wash liquid is a solution having one or more additional components (e.g., a buffer, chelator, surfactant, a detergent, a base, an acid, or a combination thereof). Exemplary solutions include, for example, a solution of 10-50 mM Tris at pH 8.0, 0.5-2 mM EDTA, and 0.5%-2% SDS, a solution of 10-50 mM Tris at pH 8.0, 0.5 to 2 mM EDTA, and 0.5%-2% Triton X-100.

The portion of channel **246** between gates **250** and **252** form a fluid reservoir **281** configured like reservoir **279** to hold a liquid (e.g., a solution) with limited or no evaporation. During operation of device **200**, the liquid of reservoir **281** is typically used as a release liquid into which polynucleotides that had been retained by particles **218** are released. An exemplary release liquid is an hydroxide solution (e.g., a NaOH solution) having a concentration of, for example, between about 2 mM hydroxide (e.g., about 2 mM NaOH) and about 500 mM hydroxide (e.g., about 500 mM NaOH). In some embodiments, liquid in reservoir **281** is an hydroxide solution having a concentration of about 25 mM or less (e.g., an hydroxide concentration of about 15 mM).

Reservoirs **279**, **281** typically hold at least about 0.375 microliters of liquid (e.g., at least about 0.750 microliters, at least about 1.25 microliters, at least about 2.5 microliters). In some embodiments, reservoirs **279**, **281** hold about 7.5 microliters or less of liquid (e.g., about 5 microliters or less, about 4 microliters or less, about 3 microliters or less).

An actuator is a component that provides a gas pressure that can move material (e.g., sample material and/or reagent material) between one location of network **201** and another location. For example, referring to FIG. 3, actuator **244** includes a chamber **272** having a mass **273** of thermally expansive material (TEM) therein. When heated, the TEM expands decreasing the free volume within chamber **272** and pressurizing the gas (e.g., air) surrounding mass **273** within

12

chamber **272**. Typically, gates **246** and **242** are actuated with actuator **244**. Consequently, the pressurized gas drives liquid in fluid reservoir **279** towards junction **255**. In some embodiments, actuator **244** can generate a pressure differential of more than about 3 psi (e.g., at least about 4 psi, at least about 5 psi) between the actuator and junction **255**.

The TEM includes a plurality of sealed liquid reservoirs (e.g., spheres) **275** dispersed within a carrier **277**. Typically, the liquid is a high vapor pressure liquid (e.g., isobutane and/or isopentane) sealed within a casing (e.g., a polymeric casing formed of monomers such as vinylidene chloride, acrylonitrile and methymethacrylate). Carrier **277** has properties (e.g., flexibility and/or an ability to soften (e.g., melt) at higher temperatures) that permit expansion of the reservoirs **275** without allowing the reservoirs to pass along channel **240**. In some embodiments, carrier **277** is a wax (e.g., an olefin) or a polymer with a suitable glass transition temperature. Typically, the reservoirs make up at least about 25 weight percent (e.g., at least about 35 weight percent, at least about 50 weight percent) of the TEM. In some embodiments, the reservoirs make up about 75 weight percent or less (e.g., about 65 weight percent or less, about 50 weight percent or less) of the TEM. Suitable sealed liquid reservoirs can be obtained from Expancel (Akzo Nobel).

When the TEM is heated (e.g., to a temperature of at least about 50° C. (e.g., to at least about 75° C., at least about 90° C.)), the liquid vaporizes and increases the volume of each sealed reservoir and of mass **273**. Carrier **277** softens allowing mass **273** to expand. Typically, the TEM is heated to a temperature of less than about 150° C. (e.g., about 125° C. or less, about 110° C. or less, about 100° C. or less) during actuation. In some embodiments, the volume of the TEM expands by at least about 5 times (e.g., at least about 10 times, at least about 20 times, at least about 30 times).

A hydrophobic vent (e.g., vent **212**) is a structure that permits gas to exit a channel while limiting (e.g., preventing) liquid from exiting the channel. Typically, hydrophobic vents include a layer of porous hydrophobic material (e.g., a porous filter such as a porous hydrophobic membrane from Osmonics) that defines a wall of the channel. As discussed below, hydrophobic vents can be used to position a microdroplet of sample at a desired location within network **201**.

The hydrophobic vents of the present invention are preferably constructed so that the amount of air that escapes through them is maximized while minimizing the volume of the channel below the vent surface. Accordingly, it is preferable that the vent is constructed so as to have a hydrophobic membrane of large surface area and a shallow cross section of the microchannel below the vent surface.

Hydrophobic vents typically have a length of at least about 2.5 mm (e.g., at least about 5 mm, at least about 7.5 mm) along a channel. The length of the hydrophobic vent is typically at least about 5 times (e.g., at least about 10 times, at least about 20 times) larger than a depth of the channel within the hydrophobic vent. For example, in some embodiments, the channel depth within the hydrophobic vent is about 300 microns or less (e.g., about 250 microns or less, about 200 microns or less, about 150 microns or less).

The depth of the channel within the hydrophobic vent is typically about 75% or less (e.g., about 65% or less, about 60% or less) of than the depth of the channel upstream and downstream of the hydrophobic vent. For example, in some embodiments the channel depth within the hydrophobic vent is about 150 microns and the channel depth upstream and downstream of the hydrophobic vent is about 250 microns.

A width of the channel within the hydrophobic vent is typically at least about 25% wider (e.g., at least about 50%

US 10,494,663 B1

13

wider) than a width of the channel upstream from the vent and downstream from the vent. For example, in an exemplary embodiment, the width of the channel within the hydrophobic vent is about 400 microns and the width of the channel upstream and downstream from the vent is about 250 microns.

Microfluidic device 200 can be fabricated as desired. Typically, layers 205, 207, and 209 are formed of a polymeric material. Components of network 201 are typically formed by molding (e.g., by injection molding) layers 207, 209. Layer 205 is typically a flexible polymeric material (e.g., a laminate) that is secured (e.g., adhesively and/or thermally) to layer 207 to seal components of network 201. Layers 207 and 209 may be secured to one another using adhesive.

In use, device 200 is typically thermally associated with an array of heat sources configured to operate the components (e.g., valves, gates, actuators, and processing region 220) of the device. In some embodiments, the heat sources are operated by an operating system, which operates the device during use. The operating system includes a processor (e.g., a computer) configured to actuate the heat sources according to a desired protocol. Processors configured to operate microfluidic devices are described in U.S. application Ser. No. 09/819,105, filed Mar. 28, 2001, which application is incorporated herein by reference. In other embodiments, the heat sources are integral with the device itself.

Device 200 may be operated as follows. Valves of network 201 are configured in the open state. Gates of network 201 are configured in the closed state. A fluidic sample comprising polynucleotides is introduced to network 201 via inlet 202. For example, sample can be introduced with a syringe having a Luer fitting. The syringe provides pressure to initially move the sample within network 201. Sample passes along channels 204, 257, 261, and 214 to inlet 265 of processing region 220. The sample passes through processing region 220, exits via outlet 267, and passes along channel 228 to waste chamber 232. When the trailing edge (e.g., the upstream liquid-gas interface) of the sample reaches hydrophobic vent 212, pressure provided by the introduction device (e.g., the syringe) is released from network 201 stopping further motion of the sample.

Typically, the amount of sample introduced is about 500 microliters or less (e.g., about 250 microliters or less, about 100 microliters or less, about 50 microliters or less, about 25 microliters or less, about 10 microliters or less). In some embodiments, the amount of sample is about 2 microliters or less (e.g., of about 0.5 microliters or less).

Polynucleotides entering processing region 220 pass through interstices between the particles 218. Polynucleotides of the sample contact retention member 216 and are preferentially retained as compared to liquid of the sample and certain other sample components (e.g., inhibitors). Typically, retention member 220 retains at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region 220. Liquid of the sample and inhibitors present in the sample exit the processing region 220 via outlet 267 and enter waste chamber 232. Processing region 220 is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during introduction of the sample.

Processing continues by washing retention member 216 with liquid of reservoir 279 to separate remaining inhibitors from polynucleotides retained by retention member 216. To wash retention member 216, valve 206 is closed and gates 242, 246 of first reservoir 240 are opened. Actuator 244 is

14

actuated and moves wash liquid within reservoir 279 along channels 257, 261, and 214, through processing region 220, and into waste reservoir 232. The wash liquid moves sample that may have remained within channels 204, 257, 261, and 214 through the processing region and into waste chamber 232. Once the trailing edge of the wash liquid reaches vent 212, the gas pressure generated by actuator 244 is vented and further motion of the liquid is stopped.

The volume of wash liquid moved by actuator 244 through processing region 220 is typically at least about 2 times the void volume of processing region 220 (e.g., at least about 3 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less). Processing region is typically at a temperature of about 50° C. or less (e.g., 30° C. or less) during washing. Exemplary wash fluids include liquids discussed with respect to reservoirs 279 and 281.

Processing continues by releasing polynucleotides from retention member 216. Typically, wash liquid from reservoir 279 is replaced with release liquid (e.g., an hydroxide solution) from reservoir 281 before releasing the polynucleotides. Valve 208 is closed and gates 250, 252 are opened. Actuator 248 is actuated thereby moving release liquid within reservoir 281 along channels 261, 214 and into processing region 220 and in contact with retention member 216. When the trailing edge of release liquid from reservoir 281 reaches hydrophobic vent 212, pressure generated by actuator 248 is vented stopping the further motion of the liquid. The volume of liquid moved by actuator 248 through processing region 220 is typically at least about equal to the void volume of the processing region 220 (e.g., at least about 2 times the void volume) and can be about 10 times the void volume or less (e.g., about 5 times the void volume or less).

Once retention member 216 with retained polynucleotides has been contacted with liquid from reservoir 281, a releasing step is typically performed. Typically, the releasing step includes heating release liquid present within processing region 216. Generally, the liquid is heated to a temperature insufficient to boil liquid in the presence of the retention member. In some embodiments, the temperature is 100° C. or less (e.g., less than 100° C., about 97° C. or less). In some embodiments, the temperature is about 65° C. or more (e.g., about 75° C. or more, about 80° C. or more, about 90° C. or more). In some embodiments, the temperature maintained for about 1 minute or more (e.g., about 2 minutes or more, about 5 minutes or more, about 10 minutes or more). In some embodiments, the temperature is maintained for about 30 minutes (e.g., about 15 minutes or less, about 10 minutes or less, about 5 minutes or less). In an exemplary embodiment, processing region 220 is heated to between about 65 and 90° C. (e.g., to about 70° C.) for between about 1 and 7 minutes (e.g., for about 2 minutes).

The polynucleotides are released into the liquid present in the processing region 220 (e.g., the polynucleotides are typically released into an amount of release liquid having a volume about the same as the void volume of the processing region 220). Typically, the polynucleotides are released into about 10 microliters or less (e.g., about 5 microliters or less, about 2.5 microliters or less) of liquid.

In certain embodiments, the ratio of the volume of original sample moved through the processing region 220 to the volume of liquid into which the polynucleotides are released is at least about 10 (e.g., at least about 50, at least about 100, at least about 250, at least about 500, at least about 1000). In some embodiments, polynucleotides from a sample having a volume of about 2 ml can be retained within the processing region, and released into about 4 microliters or

US 10,494,663 B1

15

less (e.g., about 3 microliters or less, about 2 microliters or less, about 1 microliter or less) of liquid.

The liquid into which the polynucleotides are released typically includes at least about 50% (e.g., at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region 220. The concentration of polynucleotides present in the release liquid may be higher than in the original sample because the volume of release liquid is typically less than the volume of the original liquid sample moved through the processing region. For example the concentration of polynucleotides in the release liquid may be at least about 10 times greater (e.g., at least about 25 times greater, at least about 100 times greater) than the concentration of polynucleotides in the sample introduced to device 200. The concentration of inhibitors present in the liquid into which the polynucleotides are released is generally less than concentration of inhibitors in the original fluidic sample by an amount sufficient to increase the amplification efficiency for the polynucleotides.

The time interval between introducing the polynucleotide containing sample to processing region 220 and releasing the polynucleotides into the release liquid is typically about 15 minutes or less (e.g., about 10 minutes or less, about 5 minutes or less).

Liquid including the released polynucleotides may be removed from the processing region 220 as follows. Valves 210 and 234 are closed. Gates 238 and 258 are opened. Actuator 254 is actuated to generate pressure that moves liquid and polynucleotides from processing region 220, into channel 230, and toward outlet 236. The liquid with polynucleotides can be removed using, for example, a syringe or automated sampling device. Depending upon the liquid in contact with retention member 216 during polynucleotide release, the solution with released polynucleotide may be neutralized with an amount of buffer (e.g., an equal volume of 25-50 mM Tris-HCl buffer pH 8.0).

While releasing the polynucleotides has been described as including a heating step, the polynucleotides may be released without heating. For example, in some embodiments, the liquid of reservoir 281 has an ionic strength, pH, surfactant concentration, composition, or combination thereof that releases the polynucleotides from the retention member.

While the polynucleotides have been described as being released into a single volume of liquid present within processing region 220, other configurations can be used. For example, polynucleotides may be released with the concomitant (stepwise or continuous) introduction of fluid into and/or through processing region 220. In such embodiments, the polynucleotides may be released into liquid having a volume of about 10 times or less (e.g., about 7.5 times or less, about 5 times or less, about 2.5 times or less, about 2 times or less) than the void volume of the processing region 220.

While reservoirs 279, 281 have been described as holding liquids between first and second gates, other configurations can be used. For example, liquid for each reservoir may be held within a pouch (e.g., a blister pack) isolated from network 201 by a generally impermeable membrane. The pouch is configured so that a user can rupture the membrane driving liquid into reservoirs 279, 281 where actuators 244, 248 can move the liquid during use.

While processing regions have been described as having microliter scale dimensions, other dimensions can be used. For example, processing regions with surfaces (e.g., particles) configured to preferentially retain polynucleotides as

16

opposed to inhibitors may have large volumes (e.g., many tens of microliters or more, at least about 1 milliliter or more). In some embodiments, the processing region has a bench-top scale.

While processing region 220 has been described as having a retention member formed of multiple surface-modified particles, other configurations can be used. For example, in some embodiments, processing region 220 includes a retention member configured as a porous member (e.g., a filter, a porous membrane, or a gel matrix) having multiple openings (e.g., pores and/or channels) through which polynucleotides pass. Surfaces of the porous member are modified to preferentially retain polynucleotides. Filter membranes available from, for example, Osmonics, are formed of polymers that may be surface-modified and used to retain polynucleotides within processing region 220. In some embodiments, processing region 220 includes a retention member configured as a plurality of surfaces (e.g., walls or baffles) through which a sample passes. The walls or baffles are modified to preferentially retain polynucleotides.

While processing region 220 has been described as a component of a microfluidic network, other configurations can be used. For example, in some embodiments, the retention member can be removed from a processing region for processing elsewhere. For example, the retention member may be contacted with a mixture comprising polynucleotides and inhibitors in one location and then moved to another location at which the polynucleotides are removed from the retention member.

While reservoirs 275 have been shown as dispersed within a carrier, other configurations may be used. For example, reservoirs 275 can be encased within a flexible enclosure (e.g., a membrane, for example, an enclosure such as a sack). In some embodiments, reservoirs are loose within chamber 272. In such embodiments, actuator 244 may include a porous member having pores too small to permit passage of reservoirs 275 but large enough to permit gas to exit chamber 272.

Microfluidic devices with various components are described in U.S. provisional application No. 60/553,553 filed Mar. 17, 2004 by Parunak et al., which application is incorporated herein by reference.

While microfluidic device 300 has been described as configured to receive polynucleotides already released from cells, microfluidic devices can be configured to release polynucleotides from cells (e.g., by lysing the cells). For example, referring to FIGS. 4, 5, 6A, and 6B, a microfluidic device 300 includes a sample lysing chamber 302 in which cells are lysed to release polynucleotides therein. Microfluidic device 300 further includes substrate layers L1-L3, a microfluidic network 304 (only portions of which are seen in FIG. 4), and liquid reagent reservoirs R1-R4. Liquid reagent reservoirs R1-R4 hold liquid reagents (e.g., for processing sample material) and are connected to network 304 by reagent ports RP1-RP4.

Network 304 is substantially defined between layers L2 and L3 but extends in part between all three layers L1-L3. Microfluidic network 304 includes multiple components including channels Ci, valves Vi, double valves Vi', gates Gi, mixing gates MG_i, vents Hi, gas actuators (e.g., pumps) Pi, a first processing region B1, a second processing region B2, detection zones Di, air vents AV_i, and waste zones Wi.

Components of network 304 are typically thermally actuated. As seen in FIG. 7, a heat source network 312 includes heat sources (e.g., resistive heat sources) having locations that correspond to components of microfluidic network 304. For example, the locations of heat sources HP_i correspond

US 10,494,663 B1

17

to the locations of actuators P_i , the locations of heat sources HG_i correspond to locations of gates G_i and mixing gates MG_i , the locations of heat sources HV_i correspond to the locations of valves V_i and double valves V'_i , and the locations of heat sources HD_i correspond to the locations of processing chambers D_i of network **304**. In use, the components of device **300** are disposed in thermal contact with corresponding heat sources of network **312**, which is typically operated using a processor as described above for device **200**. Heat source network **312** can be integral with or separate from device **300** as described for device **200**.

We next discuss components of microfluidic device **300**.

Air vents AV_i are components that allow gas (e.g., air) displaced by the movement of liquids within network **304** to be vented so that pressure buildup does not inhibit desired movement of the liquids. For example, air vent AV_2 permits liquid to move along channel C_{14} and into channel C_{16} by venting gas downstream of the liquid through vent AV_2 .

Valves V_i are components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). The valves V_i can have the same structure as valves of microfluidic device **200**.

As seen in FIGS. **8** and **9**, double valves V'_i are also components that have a normally open state allowing material to pass along a channel from a position on one side of the valve (e.g., upstream of the valve) to a position on the other side of the valve (e.g., downstream of the valve). Taking double valve V'_{11} of FIGS. **8** and **9** as an example, double valves V'_i include first and second masses **314**, **316** of a TRS (e.g., a eutectic alloy or wax) spaced apart from one another on either side of a channel (e.g., channel C_{14}). Typically, the TRS masses **314**, **316** are offset from one another (e.g., by a distance of about 50% of a width of the TRS masses or less). Material moving through the open valve passes between the first and second TRS masses **314**, **316**. Each TRS mass **314**, **316** is associated with a respective chamber **318**, **320**, which typically includes a gas (e.g., air).

The TRS masses **314**, **316** and chambers **318**, **320** of double valve V'_i are in thermal contact with a corresponding heat source HV'_{11} of heat source network **312**. Actuating heat source HV'_{11} causes TRS masses **314**, **316** to transition to a more mobile second state (e.g., a partially melted state) and increases the pressure of gas within chambers **318**, **320**. The gas pressure drives TRS masses **314**, **316** across channel C_{11} and closes valve HV'_{11} (FIG. **9**). Typically, masses **314**, **316** at least partially combine to form a mass **322** that obstructs channel C_{11} .

Returning to FIGS. **6A**, **6B**, gates G_i are components that have a normally closed state that does not allow material to pass along a channel from a position on one side of the gate to another side of the gate. Gates G_i can have the same structure as described for gates of device **200**.

As seen in FIG. **10A-10D**, mixing gates MG_i are components that allow two volumes of liquid to be combined (e.g., mixed) within network **304**. Mixing gates MG_i are discussed further below.

Actuators P_i are components that provide a gas pressure to move material (e.g., sample material and/or reagent material) between one location of network **304** and another location. Actuators P_i can be the same as actuators of device **200**. For example, each actuator P_i includes a chamber with a mass **273** of TEM that can be heated to pressurize gas within the chamber. Each actuator P_i includes a corresponding gate G_i (e.g., gate G_2 of actuator P_1) that prevents liquid

18

from entering the chamber of the actuator. The gate is typically actuated (e.g., opened) to allow pressure created in the chamber of the actuator to enter the microfluidic network.

Waste chambers W_i are components that can receive waste (e.g., overflow) liquid resulting from the manipulation (e.g., movement and/or mixing) of liquids within network **304**. Typically, each waste chamber W_i has an associated air vent that allows gas displaced by liquid entering the chamber to be vented.

First processing region **B1** is a component that allows polynucleotides to be concentrated and/or separated from inhibitors of a sample. Processing region **B1** can be configured and operated as processing region **220** of device **200**. In some embodiments, first processing region **B1** includes a retention member (e.g., multiple particles (e.g., microspheres or beads), a porous member, multiple walls) having at least one surface modified with one or more ligands as described for processing region **220**. For example, the ligand can include one or more polyamides (e.g., poly-cationic polyamides such as poly-L-lysine, poly-D-lysine, poly-DL-ornithine), or polyethyleneimine. In some embodiments, particles of the retention member are disposed in lysing chamber **302** and are moved into processing region **B1** along with sample material.

Second processing region **B2** is a component that allows material (e.g., sample material) to be combined with compounds (e.g., reagents) for determining the presence of one or more polynucleotides. In some embodiments, the compounds include one or more PCR reagents (e.g., primers, control plasmids, and polymerase enzymes). Typically, the compounds are stored within processing region as one or more lyophilized particles (e.g., pellets). The particles generally have a room temperature (e.g., about 20° C.) shelf-life of at least about 6 months (e.g., at least about 12 months). Liquid entering the second processing region **B2** dissolves (e.g., reconstitutes) the lyophilized compounds.

Typically, the lyophilized particle(s) of processing region **B2** have an average volume of about 5 microliters or less (e.g., about 4 microliters or less, about 3 microliters or less, about 2 microliters or less). In some embodiments, the lyophilized particle(s) of processing region **B2** have an average diameter of about 4 mm or less (e.g., about 3 mm or less, about 2 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 2 microliters and an average diameter of about 1.35 mm.

Lyophilized particles for determining the presence of one or more polynucleotides typically include multiple compounds. In some embodiments, the lyophilized particles include one or more compounds used in a reaction for determining the presence of a polynucleotide and/or for increasing the concentration of the polynucleotide. For example, lyophilized particles can include one or more enzymes for amplifying the polynucleotide as by PCR.

We next discuss exemplary lyophilized particles that include exemplary reagents for the amplification of polynucleotides associated with group B streptococcus (GBS) bacteria. In some embodiments, the lyophilized particles include one or more of a cryoprotectant, one or more salts, one or more primers (e.g., GBS Primer F and/or GBS Primer R), one or more probes (e.g., GBS Probe-FAM), one or more internal control plasmids, one or more specificity controls (e.g., Streptococcus pneumoniae DNA as a control for PCR of GBS), one or more PCR reagents (e.g., dNTPs and/or dUTPs), one or more blocking or bulking agents (e.g., non-specific proteins (e.g., bovine serum albumin (BSA), RNaseA, or gelatin), and a polymerase (e.g., glycerol-free

US 10,494,663 B1

19

Taq Polymerase). Of course, other components (e.g., other primers and/or specificity controls) can be used for amplification of other polynucleotides.

Cryoprotectants generally help increase the stability of the lyophilized particles and help prevent damage to other compounds of the particles (e.g., by preventing denaturation of enzymes during preparation and/or storage of the particles). In some embodiments, the cryoprotectant includes one or more sugars (e.g., one or more disaccharides (e.g., trehalose, melezitose, raffinose)) and/or one or more polyalcohols (e.g., mannitol, sorbitol).

Lyophilized particles can be prepared as desired. Typically, compounds of the lyophilized particles are combined with a solvent (e.g., water) to make a solution, which is then placed (e.g., in discrete aliquots (e.g., drops) such as by pipette) onto a chilled hydrophobic surface (e.g., a diamond film or a polytetrafluorethylene surface). In general, the temperature of the surface is reduced to near the temperature of liquid nitrogen (e.g., about -150°F . or less, about -200°F . or less, about -275°F . or less), such as by use of a cooling bath of a cryogenic agent directly underneath. It is to be noted that the solution is dispensed without contacting the cryogenic agent. The solution freezes as discrete particles. The frozen particles are subjected to a vacuum while still frozen for a pressure and time sufficient to remove the solvent (e.g., by sublimation) from the pellets.

In general, the concentrations of the compounds in the solution from which the particles are made is higher than when reconstituted in the microfluidic device. Typically, the ratio of the solution concentration to the reconstituted concentration is at least about 3 (e.g., at least about 4.5). In some embodiments, the ratio is about 6.

An exemplary solution for preparing lyophilized pellets for use in the amplification of polynucleotides indicative of the presence of GBS can be made by combining a cryoprotectant (e.g., 120 mg of trehalose as dry powder), a buffer solution (e.g., 48 microliters of a solution of 1M Tris at pH 8.4, 2.5M KCl, and 200 mM MgCl_2), a first primer (e.g., 1.92 microliters of 500 micromolar GBS Primer F (Invitrogen)), a second primer (e.g., 1.92 microliters of 500 micromolar GBS Primer R (Invitrogen)), a probe (e.g., 1.92 microliters of 250 micromolar GBS Probe-FAM (IDT/Biosearch Technologies)), an control probe (e.g., 1.92 microliters of 250 micromolar Cal Orange 560 (Biosearch Technologies)), a template plasmid (e.g., 0.6 microliters of a solution of 105 copies plasmid per microliter), a specificity control (e.g., 1.2 microliters of a solution of 10 nanograms per microliter (e.g., about 5,000,000 copies per microliter) *Streptococcus pneumoniae* DNA (ATCC)), PCR reagents (e.g., 4.8 microliters of a 100 millimolar solution of dNTPs (Epicenter) and 4 microliters of a 20 millimolar solution of dUTPs (Epicenter)), a bulking agent (e.g., 24 microliters of a 50 milligram per milliliter solution of BSA (Invitrogen)), a polymerase (e.g., 60 microliters of a 5 U per microliter solution of glycerol-free Taq Polymerase (Invitrogen/Ependorf)) and a solvent (e.g., water) to make about 400 microliters of solution. About 200 aliquots of about 2 microliters each of this solution are frozen and desolvated as described above to make 200 pellets. When reconstituted, the 200 particles make a PCR reagent solution having a total volume of about 2.4 milliliters.

As seen in FIG. 5, reagent reservoirs R_i are configured to hold liquid reagents (e.g., water, buffer solution, hydroxide solution) separated from network 304 until ready for use. Reservoirs R_1 include an enclosure 329 that defines a sealed space 330 for holding liquids. Each space 330 is separated from reagent port R_{Pi} and network 304 by a lower wall 333

20

of enclosure 329. A capping material 341 (e.g., a laminate, adhesive, or polymer layer) may overlie an upper wall of the enclosure.

A portion of enclosure 329 is formed as an actuation mechanism (e.g., a piercing member 331) oriented toward the lower wall 333 of each enclosure. When device 300 is to be used, reagent reservoirs R_i are actuated by depressing piercing member 331 to puncture wall 333. Piercing member 331 can be depressed by a user (e.g., with a thumb) or by the operating system used to operate device 300.

Wall 333 is typically formed of a material having a low vapor transmission rate (e.g., Aclar, a metallized (e.g. aluminum) laminate, a plastic, or a foil laminate) that can be ruptured or pierced. Reservoir 330 holds an amount of liquid suited for device 300. For example, the reservoir may hold up to about 200 microliters. The piercing member 331 may account for a portion (e.g., up to about 25%) of that volume.

In general, reservoirs R_i can be formed and filled as desired. For example, the upper wall of the enclosure can be sealed to the lower wall 333 (e.g., by adhesive and/or thermal sealing). Liquid can be introduced into the reservoir by, for example, an opening at the lower end of the piercing member 331. After filling, the opening can be sealed (e.g., by heat sealing through the localized application of heat or by the application of a sealing material (e.g., capping material 341)).

When wall 333 is punctured, fluid from the reservoir enters network 333. For example, as seen in FIGS. 5 and 6, liquid from reservoir R_2 enters network 304 by port RP_2 and travels along a channel C_2 . Gate G_3 prevents the liquid from passing along channel C_8 . Excess liquid passes along channel C_7 and into waste chamber W_2 . When the trailing edge of liquid from reservoir R_2 passes hydrophobic vent H_2 , pressure created within the reservoir is vented stopping further motion of the liquid. Consequently, network 304 receives an aliquot of liquid reagent having a volume defined by the volume of channel C_2 between a junction J_1 and a junction J_2 . When actuator P_1 is actuated, this aliquot of reagent is moved further within network 304. Reagent reservoirs R_1 , R_3 , and R_4 are associated with corresponding channels, hydrophobic vents, and actuators.

In the configuration shown, reagent reservoir R_1 typically holds a release liquid (e.g., a hydroxide solution as discussed above for device 200) for releasing polynucleotides retained within processing region B_1 . Reagent reservoir R_2 typically holds a wash liquid (e.g., a buffer solution as discussed above for device 200) for removing un-retained compounds (e.g., inhibitors) from processing region B_1 prior to releasing the polynucleotides. Reagent reservoir R_3 typically holds a neutralization buffer (e.g., 25-50 mM Tris-HCl buffer at pH 8.0). Reagent reservoir R_4 typically holds deionized water.

Lysing chamber 302 is divided into a primary lysing chamber 306 and a waste chamber 308. Material cannot pass from one of chambers 306, 308 into the other chamber without passing through at least a portion of network 304. Primary lysing chamber 306 includes a sample input port SP_1 for introducing sample to chamber 306, a sample output port SP_2 connecting chamber 306 to network 304, and lyophilized reagent LP that interact with sample material within chamber 306 as discussed below. Input port SP_1 includes a one way valve that permits material (e.g., sample material and gas) to enter chamber 306 but limits (e.g., prevents) material from exiting chamber 308 by port SP_1 . Typically, port SP_1 includes a fitting (e.g., a Luer fitting) configured to mate with a sample input device (e.g., a syringe) to form a gas-tight seal. Primary chamber 306

US 10,494,663 B1

21

typically has a volume of about 5 milliliters or less (e.g., about 4 milliliters or less). Prior to use, primary chamber 306 is typically filled with a gas (e.g., air).

Waste chamber 308 includes a waste portion W6 by which liquid can enter chamber 308 from network 304 and a vent 310 by which gas displaced by liquid entering chamber 308 can exit.

Lyophilized reagent particles LP of lysing chamber 302 include one or more compounds (e.g., reagents) configured to release polynucleotides from cells (e.g., by lysing the cells). For example, particles LP can include one or more enzymes configured to reduce (e.g., denature) proteins (e.g., proteinases, proteases (e.g., pronase), trypsin, proteinase K, phage lytic enzymes (e.g., PlyGBS)), lysozymes (e.g., a modified lysozyme such as ReadyLyse), cell specific enzymes (e.g., mutanolysin for lysing group B streptococci)).

In some embodiments, particles LP alternatively or additionally include components for retaining polynucleotides as compared to inhibitors. For example, particles LP can include multiple particles 218 surface modified with ligands as discussed above for device 200. Particles LP can include enzymes that reduce polynucleotides that might compete with a polynucleotide to be determined for binding sites on the surface modified particles. For example, to reduce RNA that might compete with DNA to be determined, particles LP may include an enzyme such as an RNAase (e.g., RNAaseA ISC BioExpress (Amresco)).

In an exemplary embodiment, particles LP cells include a cryoprotectant, particles modified with ligands configured to retain polynucleotides as compared to inhibitors, and one or more enzymes.

Typically, particles LP have an average volume of about 35 microliters or less (e.g., about 27.5 microliters or less, about 25 microliters or less, about 20 microliters or less). In some embodiments, the particles LP have an average diameter of about 8 mm or less (e.g., about 5 mm or less, about 4 mm or less). In an exemplary embodiment the lyophilized particle(s) have an average volume of about 20 microliters and an average diameter of about 3.5 mm.

Particles LP can be prepared as desired. Typically, the particles are prepared using a cryoprotectant and chilled hydrophobic surface as described above. For example, a solution for preparing particles LP can be prepared by combining a cryoprotectant (e.g., 6 grams of trehalose), a plurality of particles modified with ligands (e.g., about 2 milliliters of a suspension of carboxylate modified particles with poly-D-lysine ligands), a protease (e.g., 400 milligrams of pronase), an RNAase (e.g., 30 milligrams of RNAaseA (activity of 120 U per milligram), an enzyme that digests peptidoglycan (e.g., ReadyLyse (e.g., 160 microliters of a 30000 U per microliter solution of ReadyLyse)), a cell specific enzyme (e.g., mutanolysin (e.g., 200 microliters of a 50 U per microliter solution of mutanolysin), and a solvent (e.g., water) to make about 20 milliliters. About 1000 aliquots of about 20 microliters each of this solution are frozen and desolvated as described above to make 1000 pellets. When reconstituted, the pellets are typically used to make a total of about 200 milliliters of solution.

In use, device 300 can be operated as follows. Valves Vi and Vi' of network 304 are configured in the open state. Gates Gi and mixing gates MG1 of network 304 are configured in the closed state. Reagent ports R1-R4 are depressed to introduce liquid reagents into network 304 as discussed above. A sample is introduced to lysing chamber 302 via port SP1 and combined with lyophilized particles LP within primary lysing chamber 306. Typically, the sample includes

22

a combination of particles (e.g., cells) and a buffer solution. For example, an exemplary sample includes about 2 parts whole blood to 3 about parts buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% SDS). Another exemplary sample includes group B streptococci and a buffer solution (e.g., a solution of 20 mM Tris at pH 8.0, 1 mM EDTA, and 1% Triton X-100).

In general, the volume of sample introduced is smaller than the total volume of primary lysing chamber 306. For example, the volume of sample may be about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. A typical sample has a volume of about 3 milliliters or less (e.g., about 1.5 milliliters or less). A volume of gas (e.g., air) is generally introduced to primary chamber 306 along with the sample. Typically, the volume of gas introduced is about 50% or less (e.g., about 35% or less, about 30% or less) of the total volume of chamber 306. The volume of sample and gas combine to pressurize the gas already present within chamber 306. Valve 307 of port SP1 prevents gas from exiting chamber 306. Because gates G3, G4, G8, and G10 are in the closed state, the pressurized sample is prevented from entering network 304 via port SP2.

The sample dissolves particles LP in chamber 306. Reconstituted lysing reagents (e.g., ReadyLyse, mutanolysin) begin to lyse cells of the sample releasing polynucleotides. Other reagents (e.g., protease enzymes such as pronase) begin to reduce or denature inhibitors (e.g., proteins) within the sample. Polynucleotides from the sample begin to associate with (e.g., bind to) ligands of particles 218 released from particles LP. Typically, the sample within chamber 306 is heated (e.g., to at least about 50° C., to at least about 60° C.) for a period of time (e.g., for about 15 minutes or less, about 10 minutes or less, about 7 minutes or less) while lysing occurs. In some embodiments, optical energy is used at least in part to heat contents of lysing chamber 306. For example, the operating system used to operate device 300 can include a light source (e.g., a lamp primarily emitting light in the infrared) disposed in thermal and optical contact with chamber 306. Chamber 306 includes a temperature sensor TS used to monitor the temperature of the sample within chamber 306. The lamp output is increased or decreased based on the temperature determined with sensor TS.

Continuing with the operation of device 300, G2 is actuated (e.g., opened) providing a path between port SP2 of primary lysing chamber 306 and port W6 of lysing waste chamber 308. The path extends along channel C9, channel C8, through processing region B1, and channel C11. Pressure within chamber 306 drives the lysed sample material (containing lysate, polynucleotides bound to particles 218, and other sample components) along the pathway. Particles 218 (with polynucleotides) are retained within processing region B1 (e.g., by a filter) while the liquid and other components of the sample flow into waste chamber 308. After a period of time (e.g., between about 2 and about 5 minutes), the pressure in lysing chamber 306 is vented by opening gate G1 to create a second pathway between ports SP2 and W6. Double valves V1' and V8' are closed to isolate lysing chamber 302 from network 304.

Operation of device 300 continues by actuating pump P1 and opening gates G2, G3 and G9. Pump P1 drives wash liquid in channel C2 downstream of junction J1 through processing region B1 and into waste chamber W5. The wash liquid removes inhibitors and other compounds not retained by particles 218 from processing region B1. When the trailing edge of the wash liquid (e.g., the upstream interface) passes hydrophobic vent H14, the pressure from actuator P1

US 10,494,663 B1

23

vents from network **304**, stopping further motion of the liquid. Double valves **V2'** and **V9'** are closed.

Operation continues by actuating pump **P2** and opening gates **G6**, **G4** and **G8** to move release liquid from reagent reservoir **R1** into processing region **B1** and into contact with particles **218**. Air vent **AV1** vents pressure ahead of the moving release liquid. Hydrophobic vent **H6** vents pressure behind the trailing edge of the release liquid stopping further motion of the release liquid. Double valves **V6'** and **V10'** are closed.

Operation continues by heating processing region **B1** (e.g., by heating particles **218**) to release the polynucleotides from particles **218**. The particles can be heated as described above for device **200**. Typically, the release liquid includes about 15 mM hydroxide (e.g., NaOH solution) and the particles are heated to about 70° C. for about 2 minutes to release the polynucleotides from the particles **218**.

Operation continues by actuating pump **P3** and opening gates **G5** and **G10** to move release liquid from process region **B1** downstream. Air vent **AV2** vents gas pressure downstream of the release liquid allowing the liquid to move into channel **C16**. Hydrophobic vent **H8** vents pressure from upstream of the release liquid stopping further movement. Double valve **V11'** and valve **V14** are closed.

Referring to FIG. **10A-10D**, mixing gate **MG11** is used to mix a portion of release liquid including polynucleotides released from particles **218** and neutralization buffer from reagent reservoir **R3**. FIG. **10A** shows the mixing gate **MG11** region prior to depressing reagent reservoir **R3** to introduce the neutralization buffer into network **304**. FIG. **10B** shows the mixing gate **MG11** region, after the neutralization buffer has been introduced into channels **C13** and **C12**. Double valve **V13'** is closed to isolate network **304** from reagent reservoir **R3**. Double valve **V12'** is closed to isolate network **304** from waste chamber **W3**. The neutralization buffer contacts one side of a mass **324** of TRS of gate **MG11**.

FIG. **10 c** shows the mixing gate **MG11** region after release liquid has been moved into channel **C16**. The dimensions of microfluidic network **304** (e.g., the channel dimensions and the position of hydrophobic vent **H8**) are configured so that the portion of release liquid positioned between junctions **J3** and **J4** of channels **C16** and **C14** corresponds approximately to the volume of liquid in contact with particles **218** during the release step. In some embodiments, the volume of liquid positioned between junctions **J3** and **J4** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J3** and **J4** is about 1.75 microliters. Typically, the liquid between junctions **J3** and **J4** includes at least about 50% of polynucleotides (at least about 75%, at least about 85%, at least about 90%) of the polynucleotides present in the sample that entered processing region **B1**. Valve **V14** is closed to isolate network **304** from air vent **AV2**.

Before actuating mixing gate **MG11**, the release liquid at junction **J4** and the neutralization buffer at a junction **J6** between channels **C13** and **C12** are separated only by mass **324** of TRS (e.g., the liquids are not spaced apart by a volume of gas). To combine the release liquid and neutralization buffer, pump **P4** and gates **G12**, **G13**, and **MG11** are actuated. Pump **P4** drives the volume of neutralization liquid between junctions **J5** and **J6** and the volume of release liquid between junctions **J4** and **J3** into mixing channel **C15** (FIG. **10D**). Mass **324** of TRS typically disperses and/or melts allowing the two liquids to combine. The combined liquids include a downstream interface **335** (formed by junction **J3**)

24

and an upstream interface (formed by junction **J5**). The presence of these interfaces allows more efficient mixing (e.g., recirculation of the combined liquid) than if the interfaces were not present. As seen in FIG. **10D**, mixing typically begins near the interface between the two liquids. Mixing channel **C15** is typically at least about as long (e.g., at least about twice as long) as a total length of the combined liquids within the channel.

The volume of neutralization buffer combined with the release liquid is determined by the channel dimensions between junction **J5** and **J6**. Typically, the volume of combined neutralization liquid is about the same as the volume of combined release liquid. In some embodiments, the volume of liquid positioned between junctions **J5** and **J6** is less than about 5 microliters (e.g., about 4 microliters or less, about 2.5 microliters or less). In an exemplary embodiment the volume of release liquid between junctions **J5** and **J6** is about 2.25 microliters (e.g., the total volume of release liquid and neutralization buffer is about 4 microliters).

Returning to FIGS. **6A**, **6B**, the combined release liquid and neutralization buffer move along mixing channel **C15** and into channel **C32** (vented downstream by air vent **AV8**). Motion continues until the upstream interface of the combined liquids passes hydrophobic vent **H11**, which vents pressure from actuator **P4** stopping further motion of the combined liquids.

Continuing with operation of device **300**, actuator **P5** and gates **G14**, **G15** and **G17** are actuated to dissolve the lyophilized PCR particle present in second processing region **B2** in water from reagent reservoir **R4**. Hydrophobic vent **H10** vents pressure from actuator **P5** upstream of the water stopping further motion. Dissolution of a PCR-reagent pellet typically occurs in about 2 minutes or less (e.g., in about 1 minute or less). Valve **V17** is closed.

Continuing with operation of device **300**, actuator **P6** and gate **G16** are actuated to drive the dissolved compounds of the lyophilized particle from processing region **B2** into channel **C31**, where the dissolved reagents mix to form a homogenous dissolved lyophilized particle solution. Actuator **P6** moves the solution into channels **C35** and **C33** (vented downstream by air vent **AV5**). Hydrophobic vent **H9** vents pressure generated by actuator **P6** upstream of the solution stopping further motion. Valves **V18**, **V19**, **V20'**, and **V22'** are closed.

Continuing with operation of device **300**, actuator **P7** and gates **G18**, **MG20** and **G22** are actuated to combine (e.g., mix) a portion of neutralized release liquid in channel **32** between gate **MG20** and gate **G22** and a portion of the dissolved lyophilized particle solution in channel **C35** between gate **G18** and **MG20**. The combined liquids travel along a mixing channel **C37** and into detection region **D2**. An air vent **AV3** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H13**, the pressure from actuator **P7** is vented and the combined liquids are positioned within detection region **D2**.

Actuator **P8** and gates **MG2**, **G23**, and **G19** are actuated to combine a portion of water from reagent reservoir **R4** between **MG2** and gate **G23** with a second portion of the dissolved lyophilized particle solution in channel **C33** between gate **G19** and **MG2**. The combined liquids travel along a mixing channel **C41** and into detection region **D1**. An air vent **AV4** vents gas pressure downstream of the combined liquids. When the upstream interface of the combined liquids passes hydrophobic vent **H12**, the pressure from actuator **P8** is vented and the combined liquids are positioned within detection region **D1**.

Continuing with operation of device **300**, double valves **V26'** and **V27'** are closed to isolate detection region **D1** from network **304** and double valves **V24'** and **V25'** are closed to isolate detection region **D2** from network **304**. The contents of each detection region (neutralized release liquid with sample polynucleotides in detection region **D2** with PCR reagents from dissolved lyophilized particle solution and deionized water with PCR reagents from dissolved lyophilized particle solution in detection region **D1**) are subjected to heating and cooling steps to amplify polynucleotides (if present in detection region **D2**). The double valves of each detection region prevent evaporation of the detection region contents during heating. The amplified polynucleotides are typically detected using fluorescence detection.

While reservoirs have been shown as having a piercing member formed of a wall of the reservoir, other configurations are possible. For example, in some embodiments, the reservoir includes a needle-like piercing member that extends through an upper wall of the reservoir into the sealed space toward a lower wall of the reservoir. The upper wall of the reservoir may be sealed at the needle-like piercing member (e.g., with an adhesive, an epoxy). In use, the upper wall is depressed driving the piercing member through the lower wall forcing liquid in the sealed space to enter a microfluidic network.

While reservoirs have been described as including an actuation mechanism (e.g., a piercing member), other configurations are possible. For example, in some embodiments, a lower wall of the sealed space of the reservoir includes a weakened portion that overlies an opening to a microfluidic network. The lower wall material (e.g., laminate, polymer film, or foil) that overlies the opening is thick enough to prevent loss of the liquid within the sealed space but thin enough to rupture upon the application of pressure to the liquid therein. Typically, the material overlying the opening is thinner than the adjacent material. Alternatively, or in addition, the weakened material can be formed by leaving this material relatively unsupported as compared to the surrounding material of the lower wall.

While reservoirs have been described as having a sealed space formed in part by a wall of the sealed space, other configurations are possible. For example, referring to FIG. **11A**, a reservoir includes a plunger-like actuation mechanism (e.g., a piercing member **342**) and a gasket-like sealed space **343** having upper and lower layers **344**, **345** respectively (e.g., upper and lower laminate layers). Liquid is sealed between the upper and lower layers. The sealed space can be surrounded by a supporting structure **346** (e.g., a toroidal gasket) that supports the sealed space at its upper and lower peripheral surfaces.

Referring to FIG. **11B**, piercing member **342** is shown as being depressed until the piercing member **342** has pierced both the upper and lower layers bringing the liquid into communication with the microfluidic network. A vent **346** adjacent the plunger allows gas trapped between the piercing member and the upper layer of the sealed space to escape without being forced into the microfluidic network.

Referring to FIG. **11C**, piercing member **342** is shown as fully actuated. A portion of the piercing member has displaced a corresponding volume of liquid from the sealed space and introduced the predetermined volume of liquid into the microfluidic device.

While the reservoirs have been described as having a sealed space that may be stationary with respect to a piercing member, other configurations are possible. For example, FIG. **12A** illustrates a reservoir having a sealed space **347**

that is secured with (e.g., integral with) respect to an actuation mechanism having a movable member **348** (e.g., a plunger) and a piercing member **349** supported by a piercing member support **350** that are stationary with respect to the sealed space. Typically, the sealed space is defined by a cavity within the movable member and a lower wall **351** that seals liquid within the sealed space. Piercing member is configured to rupture the lower wall when the movable member is depressed. Piercing member support has a shape generally complementary to the cavity of the movable member. Piercing member support includes a channel **352** connected to a microfluidic network to allow fluid released from the enclosed space to enter the microfluidic network.

Referring to FIG. **12B**, the movable member has been depressed so that the piercing member has just ruptured the lower layer of the sealed space. Referring to FIG. **12C**, the reservoir has been fully depressed onto the piercing member and piercing member support. The volume of fluid displaced from the reservoir generally corresponds to the volume of the piercing member support that enters the enclosed space. A channel **353** allows air displaced by the moveable member to exit.

While reservoirs have been described as having a piercing member that is secured with respect to some portion of the reservoir, other configurations are possible. For example, referring to FIG. **13**, a reservoir includes an actuation mechanism **354** (e.g., a piercing member such as a needle-like piercing member) that is unsecured with respect to the reservoir. A sealed space **355** of the reservoir is defined by an upper wall **356** and includes a channel **357** extending through a portion of a substrate **361** in which a microfluidic network is defined. A lower wall **358** of the sealed space separates the sealed space from a channel **359** of the microfluidic network. The piercing member occupies the channel **357** of the sealed space so that the piercing tip **360** of the piercing member rests against the lower wall **358**. Depressing the upper wall **356** of the reservoir drives the piercing member **354** through the lower wall and forces liquid within the sealed space into the microfluidic network.

As another example, FIGS. **14A** and **14B** illustrate a reservoir including an actuation mechanism (e.g., a piercing member) that is initially secured to an interior of an upper wall of the reservoir but separates at least partially from the upper wall upon actuation of the reservoir.

As yet another example, FIGS. **15A** and **15B** illustrate a reservoir including a piercing member **364** that is initially secured to an interior **365** of an upper wall **366** of the reservoir but substantially separates (e.g., completely separates) from the upper wall upon actuation of the reservoir.

While reservoirs have been described as having an enclosed space that is fixed or otherwise integral with a portion of the reservoir, other configurations are possible. For example, referring to FIG. **16**, a reservoir includes a capsule-like enclosed space **367** defined by an outer wall **368**. The outer wall is generally formed of a material having a low vapor transmission rate. Reservoir also includes an actuation mechanism having a moveable member **369** with a piercing member **370** that pierces the enclosed space to release liquid therein. The liquid passes along a channel **372** leading to a microfluidic network. A channel **371** allows gas (e.g., air) otherwise trapped by the moveable member to exit.

While reservoirs have been described as generally overlying an inlet to a microfluidic network, other configurations are possible. For example, referring to FIG. **17**, a reservoir includes an enclosed space **373** in which liquid is stored and a connecting portion **374** connected to an inlet **376** of a microfluidic network. The enclosed space **373** and connect-

US 10,494,663 B1

27

ing portion **374** are separated by a rupturable seal **375** (e.g., a weak seal). In general, the rupturable seal **375** prevents liquid or vapor from exiting the enclosed space. However, upon the application of pressure to the liquid (e.g., by depressing a wall **377** of the enclosed space), the rupturable seal **375** ruptures allowing the liquid to pass through the weak seal to the connecting portion and into the microfluidic network **378**.

A still further embodiment of a reservoir with a piercing member is shown in FIG. **27A**, which shows a reservoir **2701** having an outer shell **2703** and a piercing element **2704** that are both made of the same piece of material. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2704** is generally conical in shape, with the apex adjacent to a membrane **2702**; its apex preferably does not exceed 0.040". The piercing element will puncture membrane **2702** and release liquid from reservoir **2701** when the outer shell is depressed. Representative dimensions are shown on FIG. **27A**. The reservoir may be constructed so that the upper surface is level, with a flat protective piece **2705** covering the base of the conical shape of piercing element **2704**.

Yet another embodiment of a reservoir with a piercing member is shown in FIG. **27B**, showing a reservoir **2711** having a single-piece outer shell **2712** and piercing element **2714**. Such a combined shell and piercing element can be formed from many processes known to one of ordinary skill in the art. Especially preferred processes are vacuum thermo-forming and injection moulding. Piercing element **2714** can be frustoconical in shape, with its narrower side adjacent to membrane **2713**. Alternatively, piercing element **2714** can comprise several separate piercing elements, arranged within a conical space. Preferably there are four such piercing elements where multiple elements are present.

It is to be understood that the dimensions of the reservoir, piercing element, shell and moulding shown in FIGS. **27A** and **27B** as decimal quantities in inches are exemplary. In particular, the dimensions are such that the shell does not collapse under its own weight and is not so as strong to prohibit depression of the piercing member when required during operation of the device.

Furthermore, the materials of the various embodiments are also chosen so that the device has a shelf-life of about a year. By this it is meant that the thickness of the various materials are such that they resist loss, through means such as diffusion, of 10% of the liquid volume contained therein over a desired shelf-life period.

Preferably the volume of the reservoir is around 150 μ L before a shell is depressed. Upon depression of a shell, the volume is preferably deformed to around half its original volume.

While devices for processing samples have been described as having a generally planar configuration, other configurations can be used. For example, referring to FIG. **18**, a device **700** configured to process a polynucleotide-containing sample, such as to prepare the sample for amplification of the polynucleotides, has a generally tube-like or vial-like configuration. Device **700** includes a sample reservoir **704**, a reagent reservoir **706**, a gas pressure generator **708**, a closure (e.g., a cap **710**), and a processing region **702** including a retention member **704** having a plurality of particles (e.g. carboxylate beads **705** surface-modified with a ligand, e.g., poly-L-lysine and/or poly-D-lysine, or poly-ethyleneimine). Retention member **705** and beads **705** may share any or all properties of retention member **216** and

28

surface-modified particles **218**. Device **700** also includes an opening **716** and a valve, e.g., a thermally actuated valve **714** for opening and closing opening **716**.

In use, a polynucleotide-containing sample is added to sample reservoir **704**. Typical sample amounts range from about 100 μ L to about 2 mL, although greater or smaller amounts may be used.

Reagent reservoir **706** may be provided to users of device **700** with pre-loaded reagent. Alternatively, device **700** may be configured so that users add reagent to device **700**. In any event, the reagents may include, e.g., NaOH solutions and/or buffer solutions such as any of such solutions discussed herein.

Once sample and, if necessary, reagent have been added to device **700**, cap **710** is closed to prevent evaporation of sample and reagent materials.

Referring also to FIG. **19**, an operator **718** is configured to operate device **700**. Operator **718** includes a first heat source **720** and a second heat source **722**. First heat source **720** heats sample present within sample reservoir **704**, such as to lyse cells of the polynucleotide-containing sample to prepare free polynucleotides.

Device **700** may also include an enzyme reservoir **712** comprising an enzyme, e.g., a protease such as pronase, configured to cleave peptide bonds of polypeptides present in the polynucleotide-containing sample. Enzyme reservoir **712** may be provided to users of device **700** with pre-loaded enzyme. Alternatively, device **700** may be configured so that users add enzyme to device **700**.

Device **700** may be used to reduce the amount of inhibitors present relative to the amount of polynucleotides to be determined. Thus, the sample is eluted through processing region **702** to contact constituents of the sample with beads **705**. Beads **705** retain polynucleotides of the sample as compared to inhibitors as described elsewhere herein. With valve **714** in the open state, sample constituents not retained in processing region **702** exit device **700** via the opening.

Once the polynucleotide-containing sample has eluted through processing region **702**, an amount of reagent, e.g., a wash solution, e.g., a buffer such as Tris-EDTA pH 8.0 with 1% Triton X 100 is eluted through processing region **702**. The wash solution is generally stored in reagent reservoir **706**, which may include a valve configured to release an amount of wash solution. The wash solution elutes remaining polynucleotide-containing sample and inhibitors without eluting retained polynucleotides.

Once inhibitors have been separated from retained polynucleotides, the polynucleotides are released from beads **705**. In some embodiments, polynucleotides are released by contacting the beads **705** with a release solution, e.g., a NaOH solution or buffer solution having a pH different from that of the wash solution. Alternatively, or in combination, beads **705** with retained polynucleotides are heated, such as by using second heat source **722** of operator **718**. When heat is used to release the polynucleotides, the release solution may be identical with the wash solution.

Gas pressure generator **708** may be used to expel an amount of release solution with released polynucleotides from device **700**. Gas pressure generator and/or operator **718** may include a heat source to heat gas present within generator **708**. The heated gas expands and provides the gas pressure to expel sample. In some embodiments, and whether or not thermally generated gas pressure is used, gas pressure generator **708** is configured to expel a predetermined volume of material. Typically, the amount of expelled

US 10,494,663 B1

29

solution is less than about 500 μL , less than about 250 μL , less than about 100 μL , less than about 50 μL , e.g., less than about 25 μL .

EXAMPLES

The following Examples are illustrative and are not intended to be limiting.

Example 1 Preparing Retention Member

Carboxylate surface magnetic beads (Sera-Mag Magnetic Carboxylate modified, Part #3008050250, Seradyn) at a concentration of about 1011 mL⁻¹ were activated for 30 minutes using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in a pH 6.1 500 mM 2-(N-Morpholinio)-ethanesulfonic acid (MES) buffer solution. Activated beads were incubated with 3000 Da or 300,000 Da average molecular weight poly-L-lysine (PLL). After 2 washes to remove unbound PLL, beads were ready for use.

Example 2 Microfluidic Device

Referring to FIGS. 20 and 21, a microfluidic device 300 was fabricated to demonstrate separation of polynucleotides from inhibitors. Device 300 comprises first and second substrate portions 302', 304', which respectively comprise first and second layers 302 a', 302 b' and 304 a', 304 b'. First and second layers 302 a', 302 b' define a channel 306' comprising an inlet 310' and an outlet 312'. First and second layers 304 a', 304 b' define a channel 308' comprising an inlet 314' and an outlet 316'. First and second substrate portions 302', 304' were mated using adhesive 324' so that outlet 312' communicated with inlet 314' with a filter 318' positioned therebetween. A portion of outlet 312' was filed with the activated beads prepared above to provide a processing region 320' comprising a retention member (the beads). A pipette 322' (FIG. 22) secured by adhesive 326' facilitated sample introduction.

In use, sample introduced via inlet 310' passed along channel and through processing region 320'. Excess sample material passed along channel 308' and exited device 300' via outlet 316'. Polynucleotides were preferentially retained by the beads as compared to inhibitors. Once sample had been introduced, additional liquids, e.g., a wash liquid and/or a liquid for use in releasing the retained polynucleotides were introduced via inlet 326'.

Example 3 Retention of DNA

Retention of polynucleotides by the poly-L-lysine modified beads of device 300' was demonstrated by preparing respective devices comprising processing regions having a volume of about 1 μL including about 1000 beads. The beads were modified with poly-L-lysine of between about 15,000 and 30,000 Da. Each processing region was filled with a liquid comprising herring sperm DNA (about 20 μL of sample with a concentration of about 20 mg/mL) thereby placing the beads and liquid in contact. After the liquid and beads had been in contact for 10 minutes, the liquid was removed from each processing region and subjected to quantitative real-time PCR to determine the amount of herring sperm DNA present in the liquid.

Two controls were performed. First, an otherwise identical processing region was packed with unmodified beads, i.e., beads that were identical with the poly-L-lysine beads

30

except for the activation and poly-L-lysine incubation steps. The liquid comprising herring sperm DNA was contacted with these beads, allowed to stand for 10 minutes, removed, and subjected to quantitative real-time PCR. Second, the liquid comprising the herring sperm DNA ("the unprocessed liquid") was subjected to quantitative real-time PCR.

Referring to FIG. 22, the first and second controls exhibited essentially identical responses indicating the presence of herring sperm DNA in the liquid contacted with the unmodified beads and in the unprocessed liquid. The liquid that had contacted the 3,000 poly-L-lysine beads exhibited a lower response indicating that the modified beads had retained substantially all of the herring sperm DNA. The PCR response of the liquid that had contacted the 300,000 Da poly-L-lysine beads exhibited an amplification response that was at least about 50% greater than for the 3,000 Da beads indicating that the lower molecular weight surface modification was more efficient at retaining the herring sperm DNA.

Example 4 Releasing DNA from Poly-L-Lysine Modified Beads

Devices having processing regions were packed with 3,000 Da poly-L-lysine modified beads. Liquid comprising polynucleotides obtained from group B streptococci (GB S) was contacted with the beads and incubated for 10 minutes as above for the herring sperm DNA. This liquid had been obtained by subjecting about 10,000 GBS bacteria in 10 μL of 20 mM Tris pH 8, 1 mM EDTA, 1% Triton X-100 buffer to thermal lysing at 97° C. for 3 min.

After 10 minutes, the liquid in contact with the beads was removed by flowing about 10 μL of wash solution (Tris-EDTA pH 8.0 with 1% Triton X 100) through the processing region. Subsequently, about 1 μL of 5 mM NaOH solution was added to the processing region. This process left the packed processing region filled with the NaOH solution in contact with the beads. The solution in contact with the beads was heated to 95° C. After 5 minutes of heating at 95° C., the solution in contact with the beads was removed by eluting the processing region with a volume of solution equal to three times the void volume of the processing region.

Referring to FIG. 23, five aliquots of solution were subjected to quantitative real-time PCR amplification. Aliquots E1, E2, and E3 each contained about 1 μL of liquid. Aliquot L corresponds to liquid of the original sample that had passed through the processing region. Aliquot W was liquid obtained from wash solution without heating. Aliquot E1 corresponds to the dead volume of device 300, about equal to the volume of channel 308. Thus, liquid of aliquot E1 was present in channel 308 and not in contact with the beads during heating. This liquid had passed through the processing region prior to heating. Aliquot E2 comprises liquid that was present within the processing region and in contact with the beads during heating. Aliquot E3 comprises liquid used to remove aliquot E2 from the processing region.

As seen in FIG. 23, more than 65% of the GBS DNA present in the initial sample was retained by and released from the beads (Aliquot E2). Aliquot E2 also demonstrates the release of more than 80% of the DNA that had been retained by the beads. Less than about 18% of the GBS DNA passed through the processing region without being cap-

US 10,494,663 B1

31

tured. The wash solution without heating comprised less than 5% of the GBS DNA (Aliquot W).

Example 5 Separation of Polynucleotides and Inhibitors

Buccal cells from the lining of the cheeks provide a source of human genetic material (DNA) that may be used for single nucleotide polymorphism (SNP) detection. A sample comprising buccal cells was subjected to thermal lysing to release DNA from within the cells. Device 300 was used to separate the DNA from concomitant inhibitors as described above. A cleaned-up sample corresponding to aliquot E2 of FIG. 23 was subjected to polymerase chain reaction. A control or crude sample as obtained from the thermal lysing was also amplified.

Referring to FIG. 24, the cleaned-up sample exhibited substantially higher PCR response in fewer cycles than did the control sample. For example, the clean-up sample exceeded a response of 20 within 32 cycles whereas the control sample required about 45 cycles to achieve the sample response.

Blood acts as a sample matrix in variety of diagnostic tests including detection of infectious disease agents, cancer markers and other genetic markers. Hemoglobin present in blood samples is a documented potent inhibitor of PCR. Two 5 ml blood samples were lysed in 20 mM Tris pH 8, 1 mM EDTA, 1% SDS buffer and introduced to respective devices 300, which were operated as described above to prepare two clean-up samples. A third 5 ml blood sample was lysed and prepared using a commercial DNA extraction method Puregene, Gentra Systems, MN. The respective cleaned-up samples and sample subjected to the commercial extraction method were used for a Allelic discrimination analysis (CYP2D6*4 reagents, Applied Biosystems, CA). Each sample contained an amount of DNA corresponding to about 1 ml of blood.

Referring to FIG. 25, the cleaned-up and commercially extracted samples exhibited similar PCR response demonstrating that the processing region of device 300' efficiently removed inhibitors from the blood samples.

Example 6 Protease Resistant Retention Member

The preparation of polynucleotide samples for further processing often includes subjecting the samples to protease treatment in which a protease cleaves peptide bonds of proteins in the sample. An exemplary protease is pronase, a mixture of endo- and exo-proteases. Pronase cleaves most peptide bonds. Certain ligands, such as poly-L-lysine are susceptible to rupture by pronase and other proteases. Thus, if samples are generally not subjected to protease treatment in the presence of the retention member if the ligands bound thereto are susceptible to the proteases.

Poly-D-lysine, the dextro enantiomer of poly-lysine resists cleavage by pronase and other proteases. The ability of a retention member comprising bound poly-D-lysine to retain DNA even when subjected to a protease treatment was studied.

Eight (8) samples were prepared. A first group of 4 samples contained 1000 GBS cells in 10 μ l buffer. A second group of 4 samples contained 100 GBS cells in 10 μ l buffer. Each of the 8 samples was heated to 97° C. for 3 min to lyse the GBS cells. Four (4) sample sets were created from the heated samples. Each sample set contained 1 sample from each of the first and second groups. The samples of each sample sets were treated as follows.

32

Referring to FIG. 26A, the samples of sample set 1 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 2 were subjected to pronase incubation to prepare respective protein cleaved samples, which were then heated to inactivate the proteases. The protein-cleaved, heated samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released DNA were neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 3 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-L-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

The samples of sample set 4 were subjected to pronase incubation to prepare respective protein cleaved samples. The proteases were not deactivated either thermally or chemically. The protein-cleaved samples were contacted with respective retention members each comprising a set of poly-D-lysine modified beads. After 5 minutes, the respective sets of beads were washed with 5 microliters of a 5 mM NaOH solution to separate inhibitors and products of protein cleavage from the bound DNA. The respective sets of beads were each contacted with a second aliquot of NaOH solution and heated to 80 (eighty)° C. for 2 minutes to release the DNA. The solutions with released polynucleotides were each neutralized with an equal volume of buffer. The neutralized solutions were analyzed to determine the efficiency of DNA recovery. The results were averaged and shown in FIG. 26B.

As seen in FIG. 26B, an average of more than 80% of DNA from the GBS cells was recovered using sample set 4 in which the samples were contacted with poly-D-lysine modified beads and subjected to pronase incubation in the presence of the beads without protease inactivation. The

US 10,494,663 B1

33

recovery efficiency for sample set 4 is more than twice as high as for any of the other samples. Specifically, the recovery efficiencies for sample sets 1, 2, 3, and 4, were 29%, 32%, 14%, and 81.5%, respectively. The efficiencies demonstrate that high recovery efficiencies can be obtained for samples subjected to protease incubation in the presence of a retention member that retains DNA.

Other embodiments are within the claims.

What is claimed is:

1. A method of isolating polynucleotides from a biological sample in a molecular diagnostic system, the method comprising:

receiving the biological sample in a lysing container in the system;

contacting the biological sample with a buffer solution and a lysing reagent in the lysing container, wherein the buffer solution has a pH of about 8.5 or less;

heating the biological sample in the lysing container to a first temperature between about 30° C. and about 50° C., wherein the polynucleotides are extracted from the biological sample into a lysate solution;

contacting the polynucleotides with a plurality of magnetic binding particles in the lysing container, the plurality of magnetic binding particles comprising polycationic molecules on the surface thereof, wherein at least a portion of the polynucleotides are retained on the plurality of magnetic binding particles in the lysate solution;

transferring the lysate solution containing the plurality of magnetic binding particles into a first processing region, wherein the first processing region is within a microfluidic network in the system, and wherein the lysing container is located external to the microfluidic network;

capturing the plurality of magnetic binding particles in the first processing region, wherein excess lysate solution flows through the first processing region into a waste chamber;

introducing a wash solution into the first processing region to remove unbound material not retained by the plurality of magnetic binding particles;

introducing a release solution into the first processing region, wherein the release solution has a pH of at least 10.5;

heating the first processing region to a second temperature greater than the first temperature, wherein at least a portion of the polynucleotides are eluted from the plurality of magnetic binding particles into an eluate solution; and

transferring the eluate solution containing polynucleotides to a second processing region in the system, wherein the eluate solution reconstitutes PCR reagents contained in the second processing region to form a PCR-ready solution.

2. The method of claim 1, wherein the second temperature is between about 80° C. and about 100° C.

3. The method of claim 1, wherein the lysate solution is heated in the lysing container for about 10 minutes.

4. The method of claim 1, wherein the polycationic molecules are covalently bound to the surfaces of the plurality of magnetic binding particles.

5. The method of claim 4, wherein the plurality of magnetic binding particles comprise one or more carboxylic groups to provide an attachment point for the polycationic molecules.

34

6. The method of claim 1, wherein the plurality of magnetic binding particles are preloaded in the lysing container prior to receiving the biological sample in the lysing container.

7. The method of claim 1, wherein the lysing container further comprises a proteinase K enzyme that is preloaded in the lysing container prior to receiving the biological sample in the lysing container.

8. The method of claim 1, further comprising treating magnetic beads with EDAC and NHS to form the plurality of magnetic binding particles.

9. The method of claim 1, wherein the polycationic molecules have a molecular weight of less than about 30,000 Da.

10. The method of claim 1, wherein the polycationic molecules have a molecular weight of less than about 800 Da.

11. The method of claim 1, wherein the PCR reagents comprise assay-specific primers and probes.

12. The method of claim 11, wherein the assay-specific primers and probes are specific to group B streptococcus (GBS) bacteria.

13. The method of claim 1, wherein the PCR reagents in the second processing region are dry PCR reagents.

14. The method of claim 13, wherein the dry PCR reagents have a room-temperature shelf life of at least 12 months.

15. The method of claim 1, wherein the second processing region further comprises sample process control reagents.

16. The method of claim 1, wherein the buffer solution used in the lysing container comprises Triton-X 100.

17. The method of claim 1, wherein the release solution introduced into the first processing region comprises NaOH.

18. The method of claim 17, wherein the release solution has a pH of at least 11.4.

19. The method of claim 17, wherein the release solution has a pH between about 11.7 and about 13.

20. The method of claim 1, further comprising applying pressure to the lysate solution to move the lysate solution through one or more channels in the microfluidic network into the first processing region.

21. The method of claim 20, wherein applying pressure to the lysate solution comprises applying air pressure through an inlet in the microfluidic network via an introduction device.

22. The method of claim 21, wherein the introduction device comprises a syringe or a pipette.

23. The method of claim 1, wherein introducing the wash solution into the first processing region comprises pumping the wash solution through one or more channels in the microfluidic network into the first processing region.

24. The method of claim 1, wherein introducing the release solution into the first processing region comprises pumping the release solution from a reagent reservoir located external to the microfluidic network through a port in the microfluidic network and through one or more channels in the microfluidic network into the first processing region.

25. The method of claim 1, further comprising removing the eluate solution from the first processing region via an automated sampling device.

26. The method of claim 1, further comprising removing the eluate solution from the first processing region via a syringe or a pipette.

27. A method of isolating polynucleotides from a biological sample in a molecular diagnostic system, the method comprising:

US 10,494,663 B1

35

receiving the biological sample in a lysing container in the system;
 contacting the biological sample with a buffer solution and a lysing reagent in the lysing container, wherein the buffer solution has a pH of about 8.5 or less;
 heating the biological sample in the lysing container to a first temperature of about 60° C., wherein the polynucleotides are extracted from the biological sample into a lysate solution;
 contacting the polynucleotides with a plurality of magnetic binding particles in the lysing container, the plurality of magnetic binding particles comprising polycationic molecules on the surface thereof, wherein at least a portion of the polynucleotides are retained on the plurality of magnetic binding particles in the lysate solution;
 transferring the lysate solution containing the plurality of magnetic binding particles into a first processing region, wherein the first processing region is within a microfluidic network in the system; and wherein the lysing container is outside of the microfluidic network;
 capturing the plurality of magnetic binding particles in the first processing region, wherein excess lysate solution flows through the first processing region into a waste chamber;
 introducing a wash solution into the first processing region to remove unbound material not retained by the plurality of magnetic binding particles;
 introducing a release solution into the first processing region, wherein the release solution has a pH of at least 10.5;
 heating the first processing region to a second temperature greater than the first temperature, wherein at least a portion of the polynucleotides are eluted from the plurality of magnetic binding particles into an eluate solution; and
 transferring the eluate solution containing polynucleotides to a second processing region in the system, wherein the eluate solution reconstitutes PCR reagents contained in the second processing region to form a PCR-ready solution.

28. The method of claim 27, wherein the second temperature is between about 80° C. and about 100° C.

29. The method of claim 27, wherein the lysate solution is heated in lysing container for about 10 minutes.

30. The method of claim 27, wherein the polycationic molecules are covalently bound to the surfaces of the plurality of magnetic binding particles.

31. The method of claim 30, wherein the plurality of magnetic binding particles comprise one or more carboxylic groups to provide an attachment point for the polycationic molecules.

32. The method of claim 27, wherein the plurality of magnetic binding particles are preloaded in the lysing container prior to receiving the biological sample in the lysing container.

33. The method of claim 27, wherein the lysing container further comprises a proteinase K enzyme that is preloaded in the lysing container prior to receiving the biological sample in the lysing container.

36

34. The method of claim 27, further comprising treating magnetic beads with EDAC and NHS to form the plurality of magnetic binding particles.

35. The method of claim 27, wherein the polycationic molecules have a molecular weight of less than about 30,000 Da.

36. The method of claim 27, wherein the polycationic molecules have a molecular weight of less than about 800 Da.

37. The method of claim 27, wherein the PCR reagents in the second processing region are dry PCR reagents.

38. The method of claim 37, wherein the dry PCR reagents have a room-temperature shelf life of at least 12 months.

39. The method of claim 27, wherein the second processing region further comprises sample process control reagents.

40. The method of claim 27, wherein the buffer solution used in the lysing container comprises Triton-X 100.

41. The method of claim 27, wherein the release solution introduced into the first processing region comprises NaOH and has a pH of at least 11.4.

42. The method of claim 41, wherein the release solution has a pH between about 11.7 and about 13.

43. The method of claim 27, further comprising applying pressure to the lysate solution to move the lysate solution through one or more channels in the microfluidic network into the first processing region.

44. The method of claim 43, wherein applying pressure to the lysate solution comprises applying pressure to an inlet in the microfluidic network via an introduction device.

45. The method of claim 44, wherein the introduction device comprises a syringe or a pipette.

46. The method of claim 27, wherein introducing the wash solution into the first processing region comprises pumping the wash solution through one or more channels in the microfluidic network into the first processing region, and wherein introducing the release solution into the first processing region comprises pumping the release solution from a reagent reservoir located external to the microfluidic network through a port in the microfluidic network and through one or more channels in the microfluidic network into the first processing region.

47. The method of claim 27, wherein introducing the release solution into the first processing region comprises pumping the release solution from a reagent reservoir located external to the microfluidic network through a port in the microfluidic network and through one or more channels in the microfluidic network into the first processing region.

48. The method of claim 27, further comprising removing the eluate solution from the first processing region via an automated sampling device.

49. The method of claim 27, further comprising removing the eluate solution from the first processing region via a syringe or a pipette.

* * * * *

EXHIBIT 47



PATENTS

Product	Patents
CARTRIDGE	US Patent Nos. 9,738,887; 9,433,940; 9,101,930; 9,403,165; and 9,452,430. AU Patent No. 2013221701. JP Patent No. 6061313.
P02 (overall system and method)	US Patent Nos. 9,050,594; 9,339,812; 9,441,219; 10,041,062; 9,604,213; and 10,010,888. CN Patent No. 201380009286.3.
EXTRACTION PLATE	US Patent Nos. 9,382,532; and 9,540,636. DE Patent No. 60 2013 056 932.0. DK Patent No. 2912174. EP Patent No. EP-B-2912174. ES Patent No. 300330453. FR Patent No. 2912174. GB Patent No. 2912174. IT Patent No. 502019000064944. NO Patent No. 2912174. SE Patent No. 2912174.
XPCR MODULE	US Patent Nos. 9,499,896; 10,239,060; 9,539,576; 10,226,771; 9,637,775; 10,093,963; 9,604,213; and 10,010,888.

** Other US and foreign patents pending.*

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EXHIBIT 48



NeuMoDx™ MOLECULAR SYSTEMS INTEGRATING THE DIAGNOSTIC PROCESS

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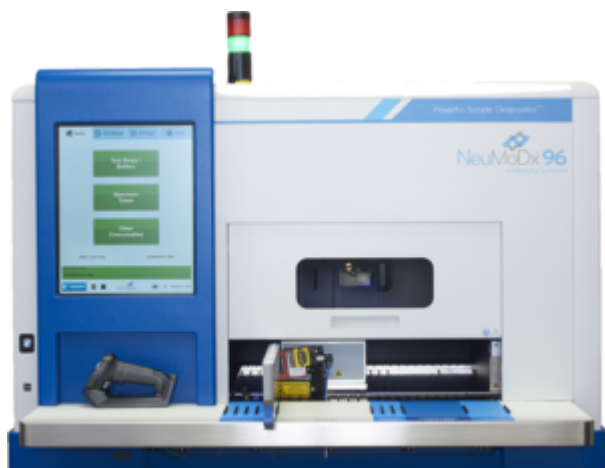
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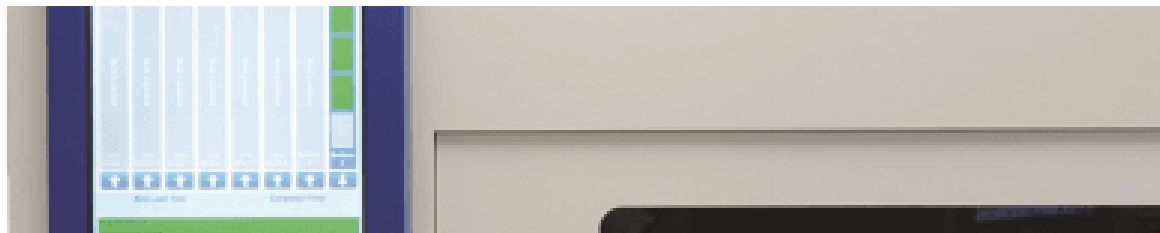
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EXHIBIT 49



US010093963B2

(12) **United States Patent**
Kusner et al.

(10) **Patent No.:** **US 10,093,963 B2**
(45) **Date of Patent:** **Oct. 9, 2018**

(54) **SYSTEM AND METHOD FOR PROCESSING BIOLOGICAL SAMPLES**

(56) **References Cited**

(71) Applicant: **NeuMoDx Molecular, Inc.**, Ann Arbor, MI (US)

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(72) Inventors: **Michael T. Kusner**, Ann Arbor, MI (US); **Jeffrey Williams**, Ann Arbor, MI (US)

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(73) Assignee: **NeuMoDx Molecular, Inc.**, Ann Arbor, MI (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(21) Appl. No.: **15/465,796**

Primary Examiner — Nathan A Bowers

(22) Filed: **Mar. 22, 2017**

Assistant Examiner — Lydia Edwards

(65) **Prior Publication Data**

US 2017/0191115 A1 Jul. 6, 2017

(74) *Attorney, Agent, or Firm* — Jeffrey Schox

Related U.S. Application Data

(63) Continuation of application No. 14/229,396, filed on Mar. 28, 2014, now Pat. No. 9,637,775, which is a (Continued)

(57) **ABSTRACT**

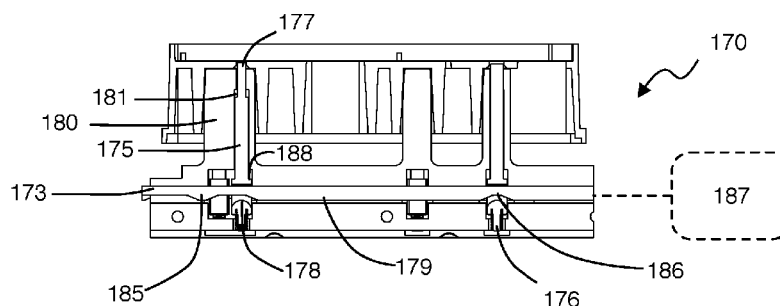
A system and method for processing and detecting nucleic acids from a set of biological samples, comprising: a molecular diagnostic module configured to receive nucleic acids bound to magnetic beads, isolate nucleic acids, and analyze nucleic acids, comprising a cartridge receiving module, a heating/cooling subsystem and a magnet configured to facilitate isolation of nucleic acids, a valve actuation subsystem including an actuation substrate, and a set of pins interacting with the actuation substrate, and a spring plate configured to bias at least one pin in a configurations, the valve actuation subsystem configured to control fluid flow through a microfluidic cartridge for processing nucleic acids, and an optical subsystem for analysis of nucleic acids; and a fluid handling system configured to deliver samples and reagents to components of the system to facilitate molecular diagnostic protocols.

(51) **Int. Cl.**
C12Q 1/6806 (2018.01)
B01L 3/00 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC **C12Q 1/6806** (2013.01); **B01L 3/502715** (2013.01); **B01L 3/502738** (2013.01);
(Continued)

(58) **Field of Classification Search**
CPC B29C 66/71; B29C 65/08; B29C 65/606; B29C 66/81423; B29C 66/8322;
(Continued)

20 Claims, 11 Drawing Sheets



US 10,093,963 B2

Page 2

Related U.S. Application Data

- continuation-in-part of application No. 13/766,359, filed on Feb. 13, 2013, now Pat. No. 9,050,594.
- (60) Provisional application No. 61/667,606, filed on Jul. 3, 2012, provisional application No. 61/598,240, filed on Feb. 13, 2012.
- (51) **Int. Cl.**
F16K 99/00 (2006.01)
B01L 99/00 (2010.01)
- (52) **U.S. Cl.**
CPC **F16K 99/0005** (2013.01); **B01L 2200/025** (2013.01); **B01L 2200/027** (2013.01); **B01L 2200/0631** (2013.01); **B01L 2200/10** (2013.01); **B01L 2300/0654** (2013.01); **B01L 2300/0681** (2013.01); **B01L 2300/087** (2013.01); **B01L 2300/123** (2013.01); **F16K 2099/0084** (2013.01)
- (58) **Field of Classification Search**
CPC B29K 2021/00; B01L 2200/0684; B01L 2200/0689; B01L 2200/10; B01L 2300/1827; B01L 2400/0487; B01L 2400/0655; B01L 3/5027
- See application file for complete search history.

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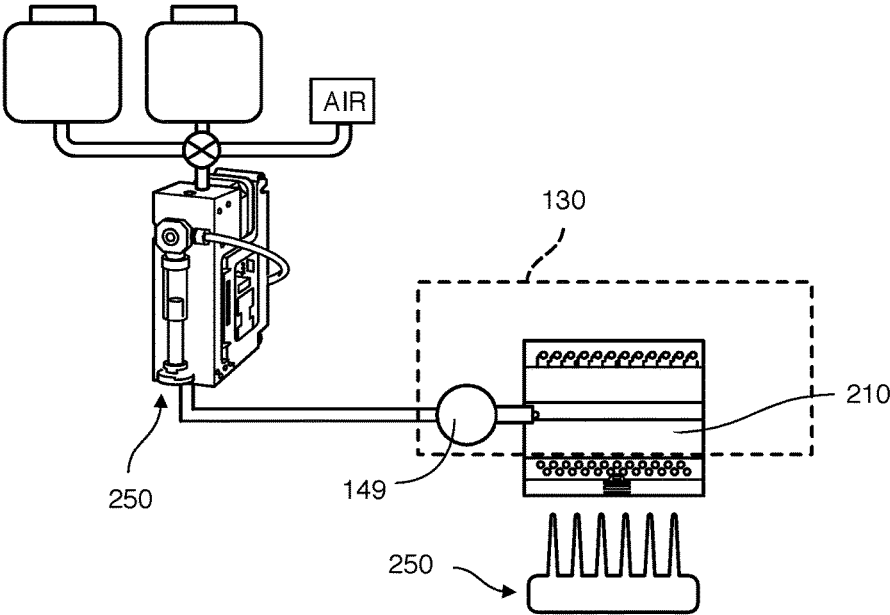


FIGURE 1A

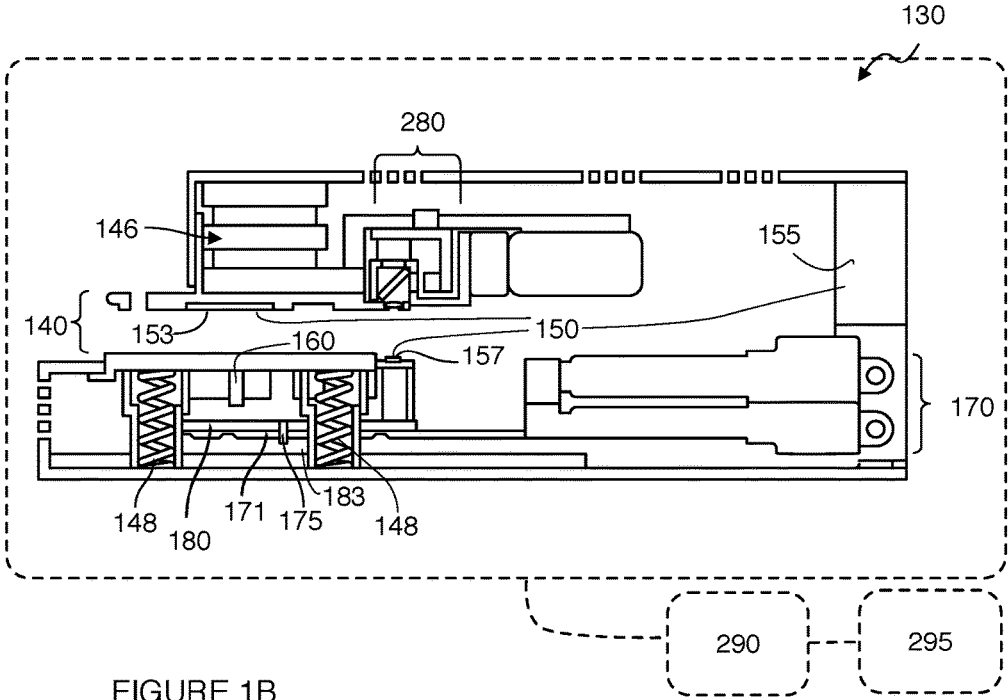


FIGURE 1B

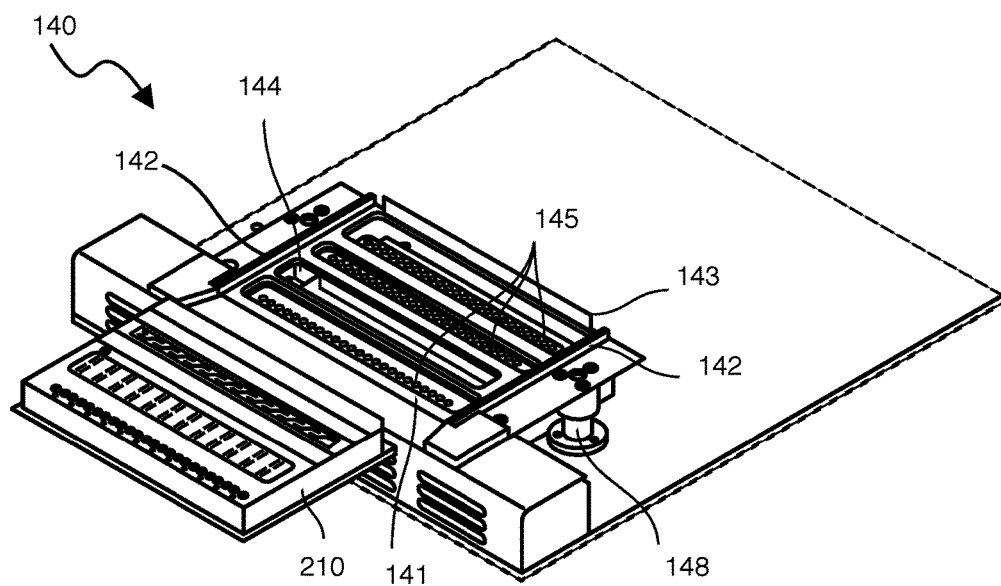


FIGURE 2

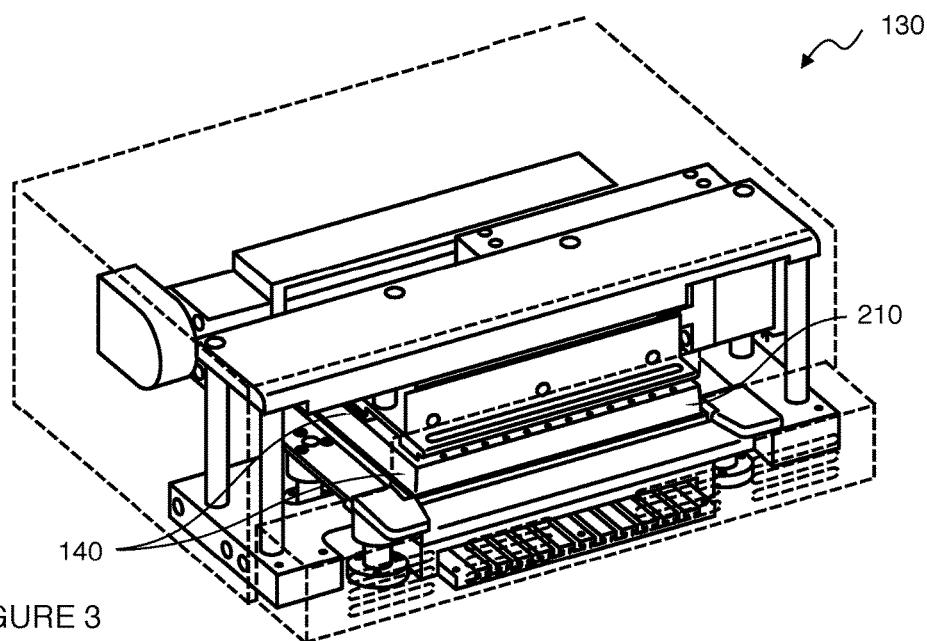


FIGURE 3

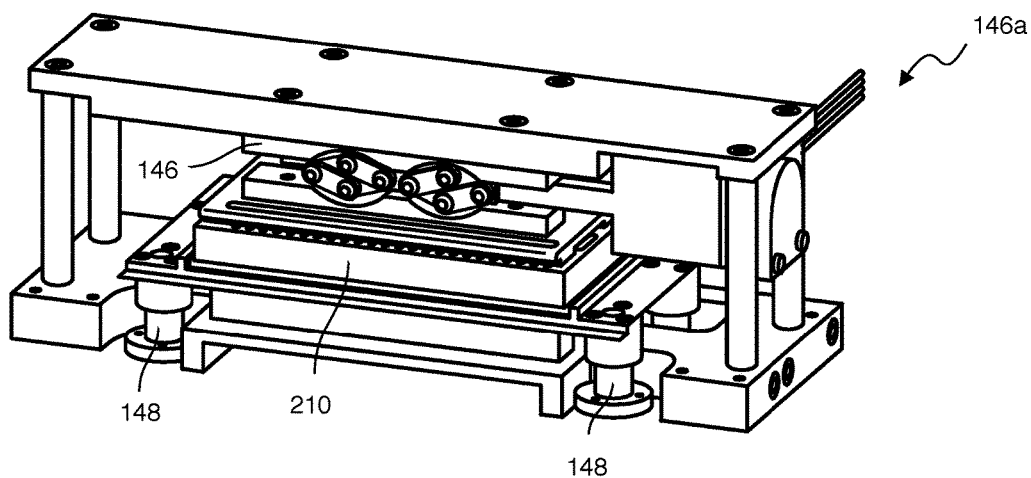


FIGURE 4A

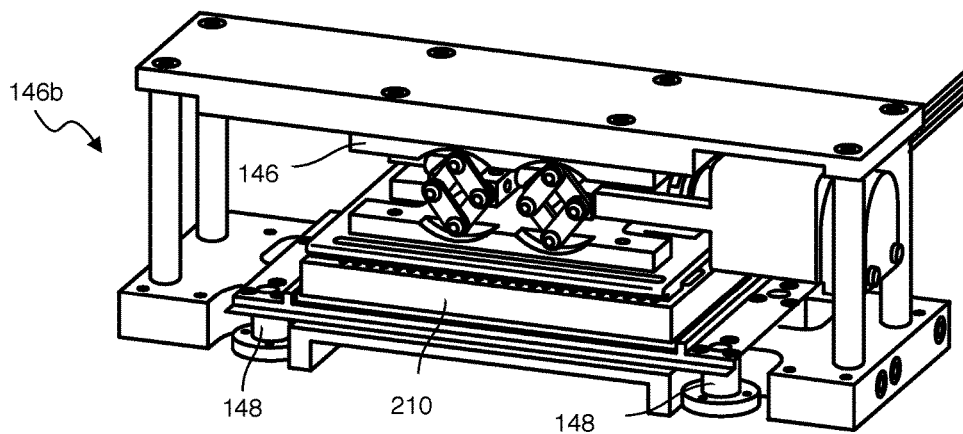


FIGURE 4B

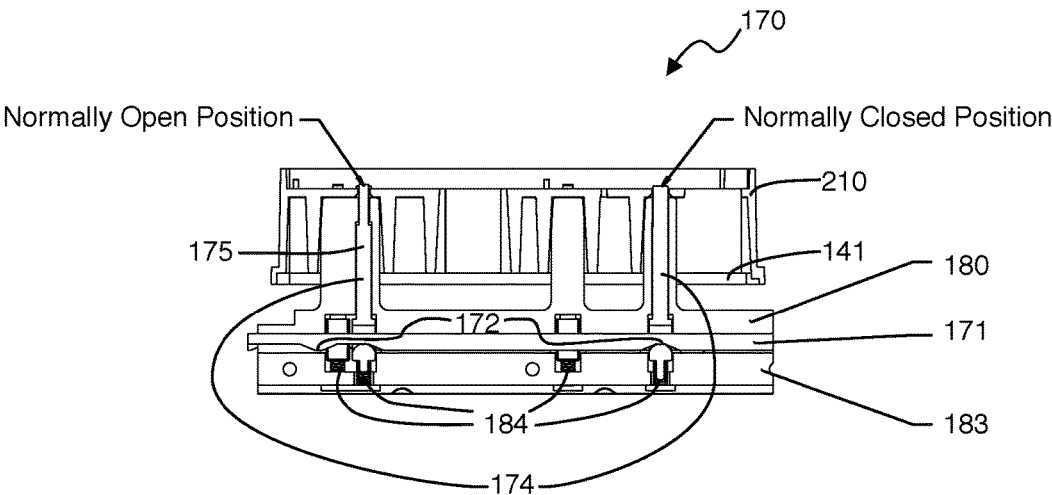


FIGURE 5A

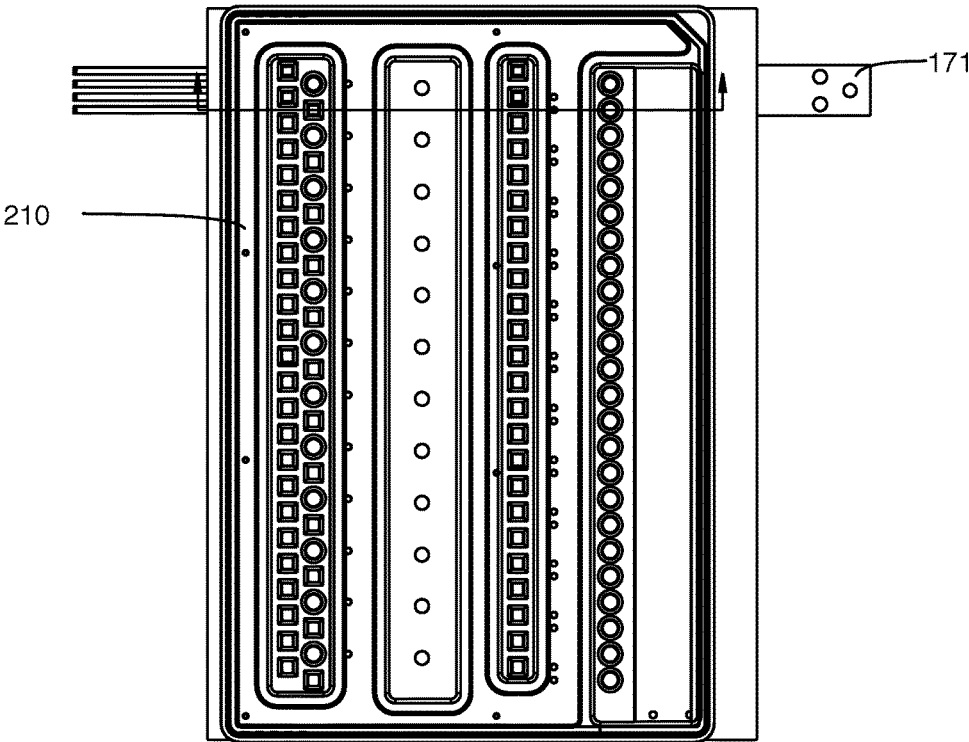
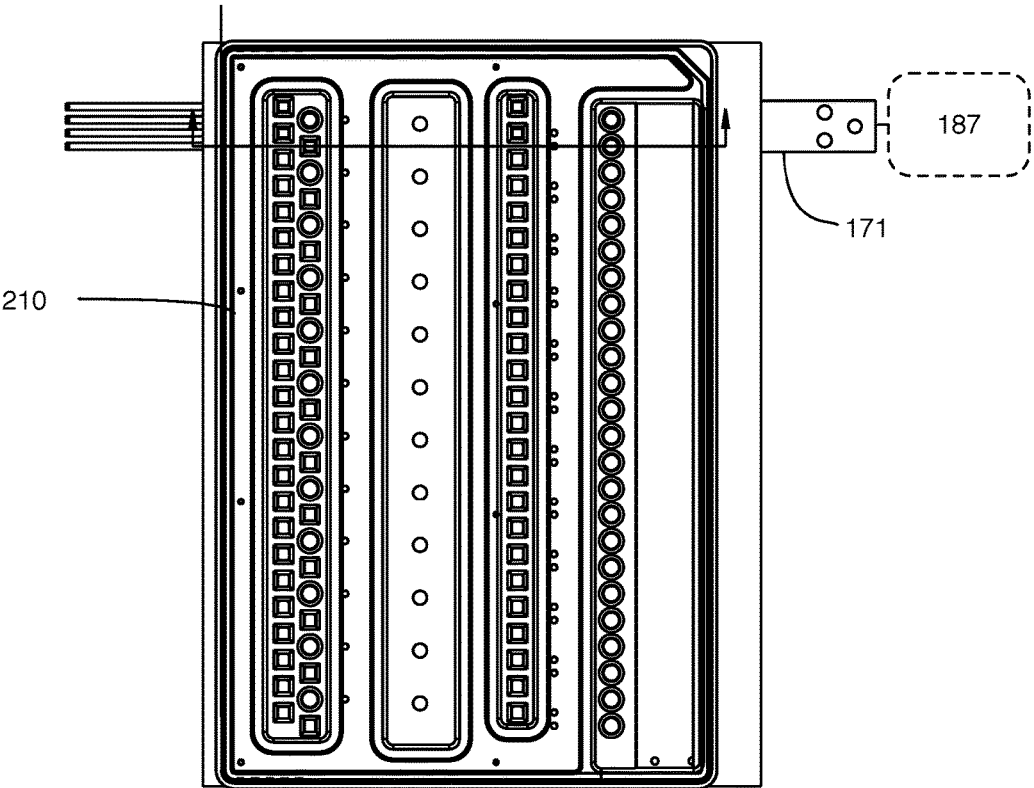
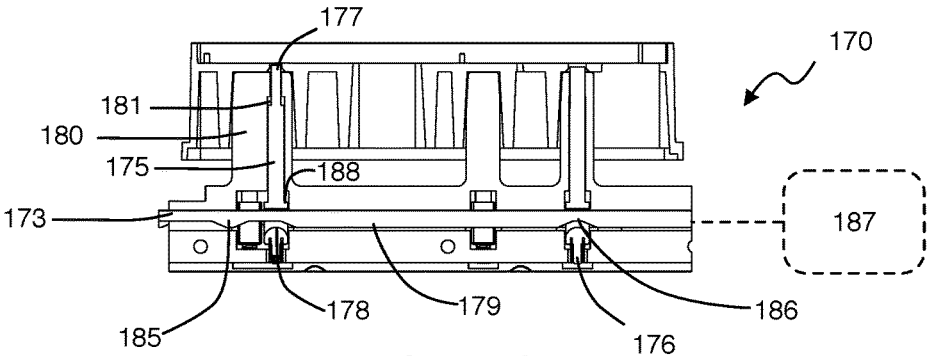


FIGURE 5B



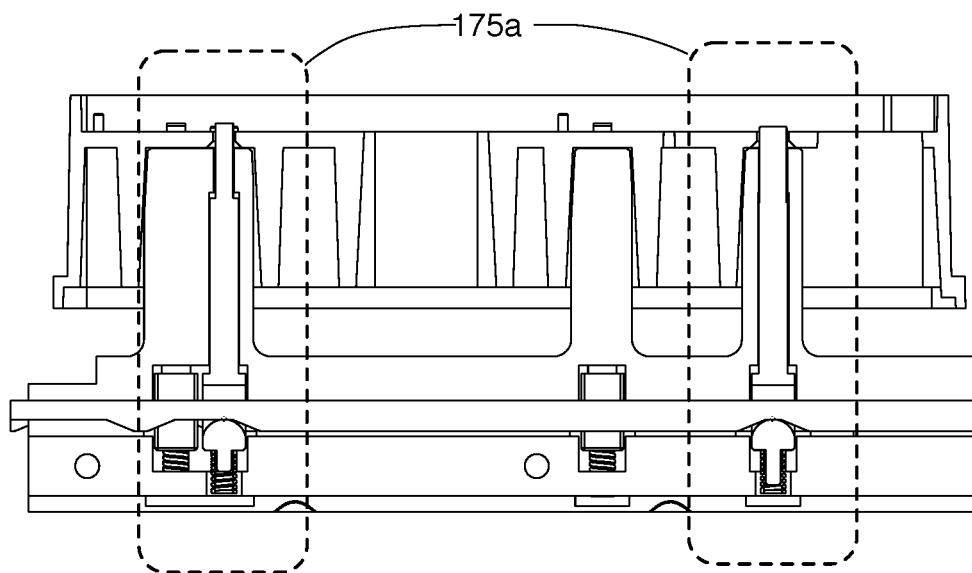


FIGURE 6A

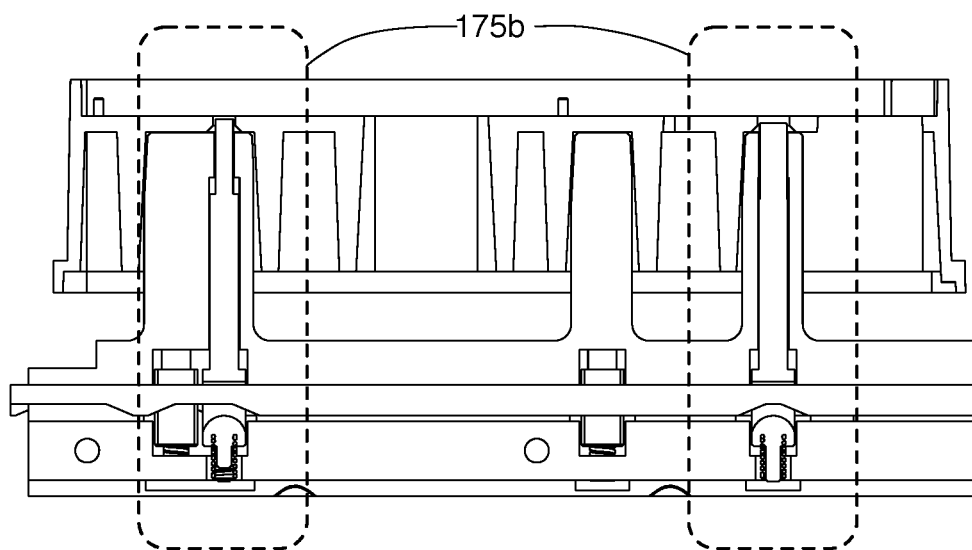
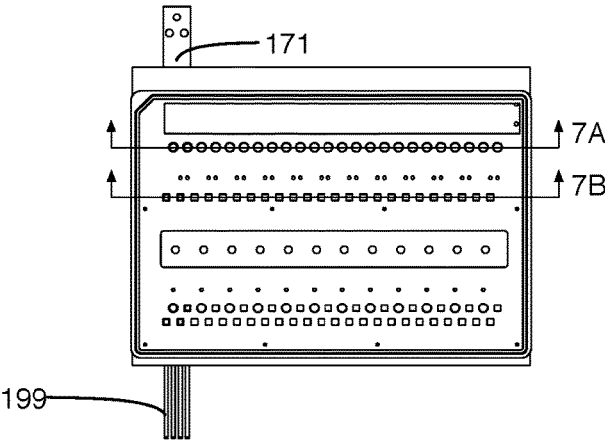
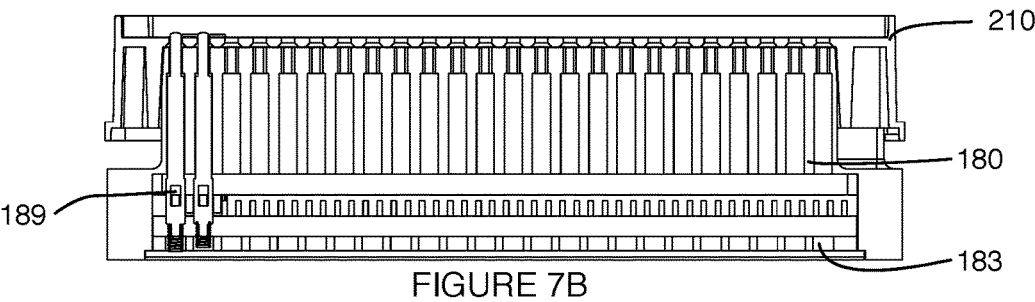
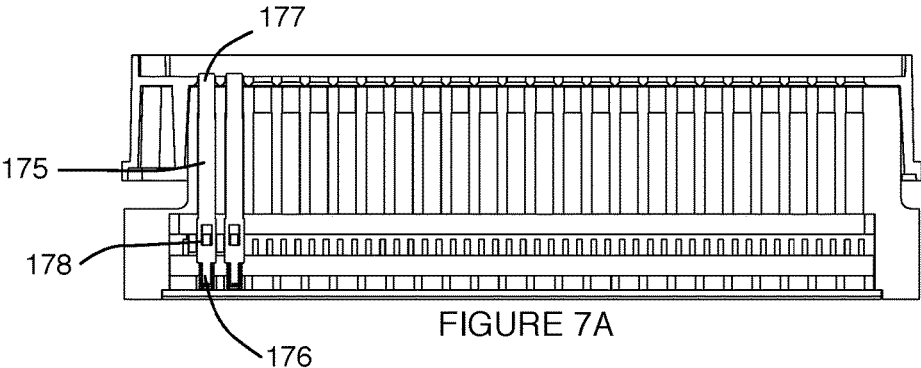


FIGURE 6B



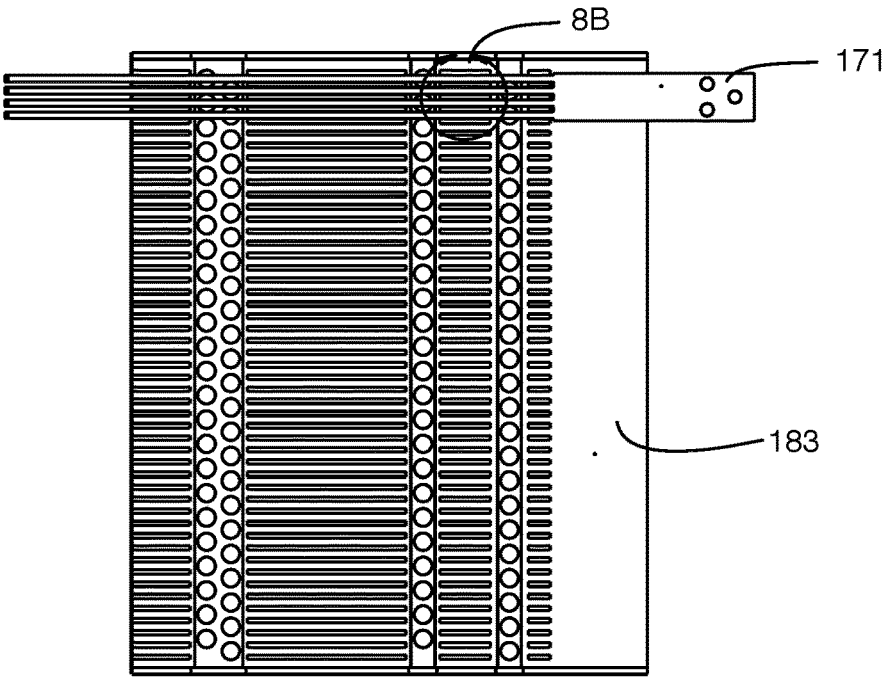


FIGURE 8A

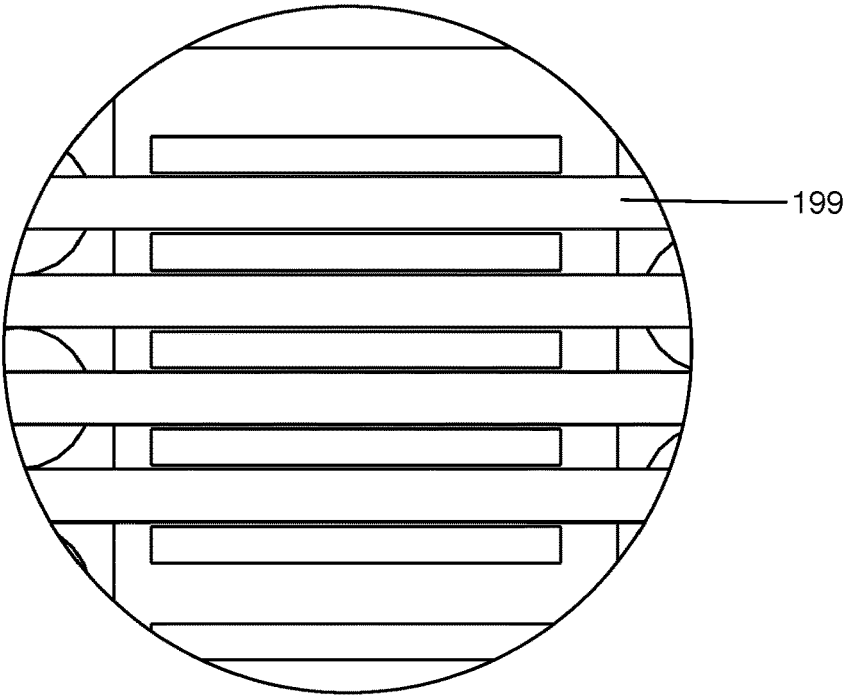
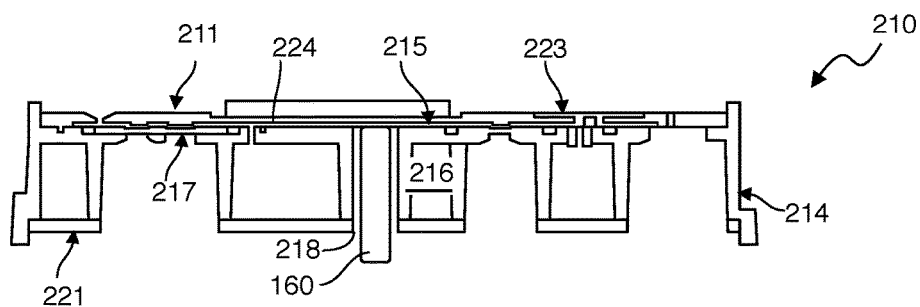
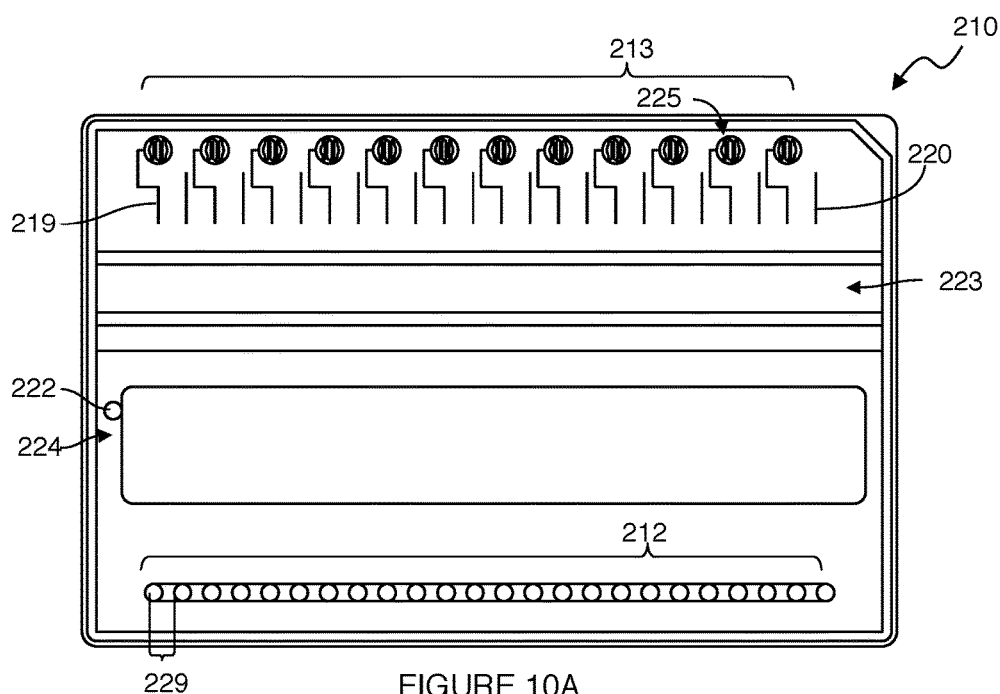
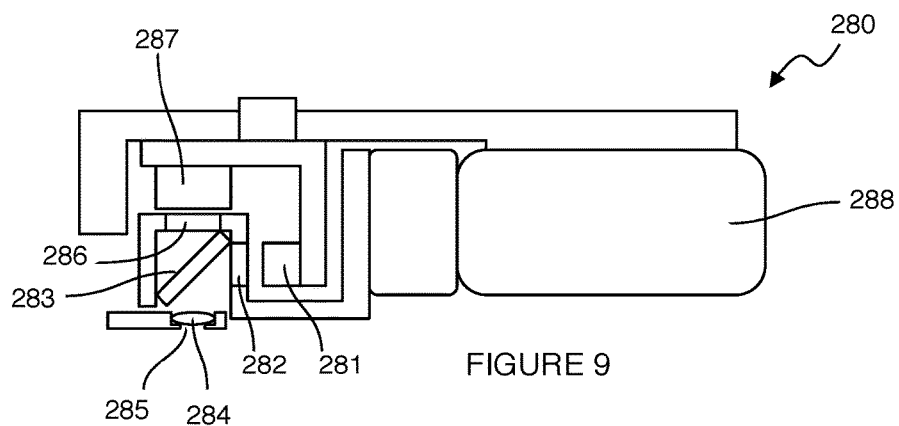


FIGURE 8B



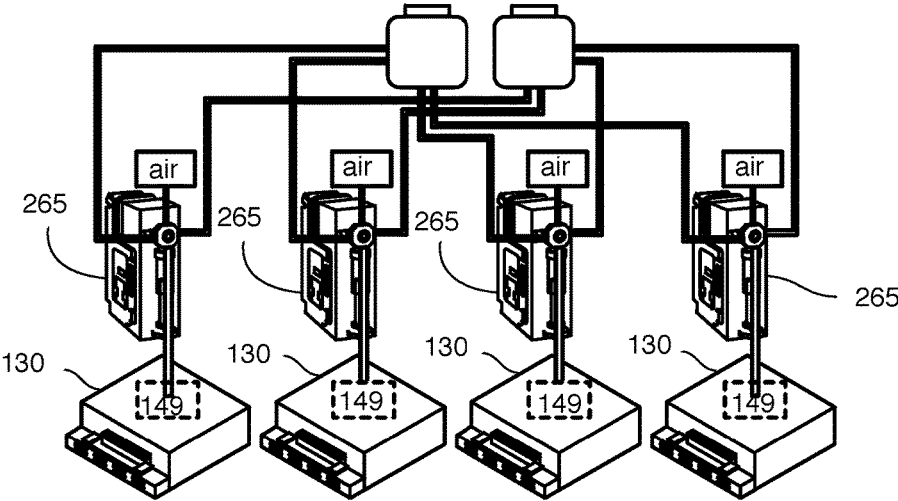


FIGURE 11A

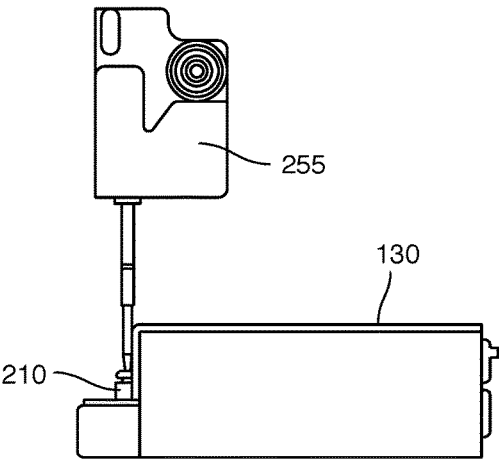


FIGURE 11B

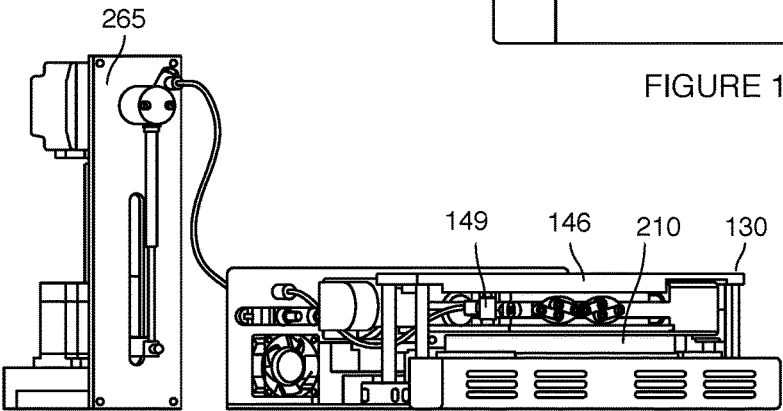


FIGURE 11C

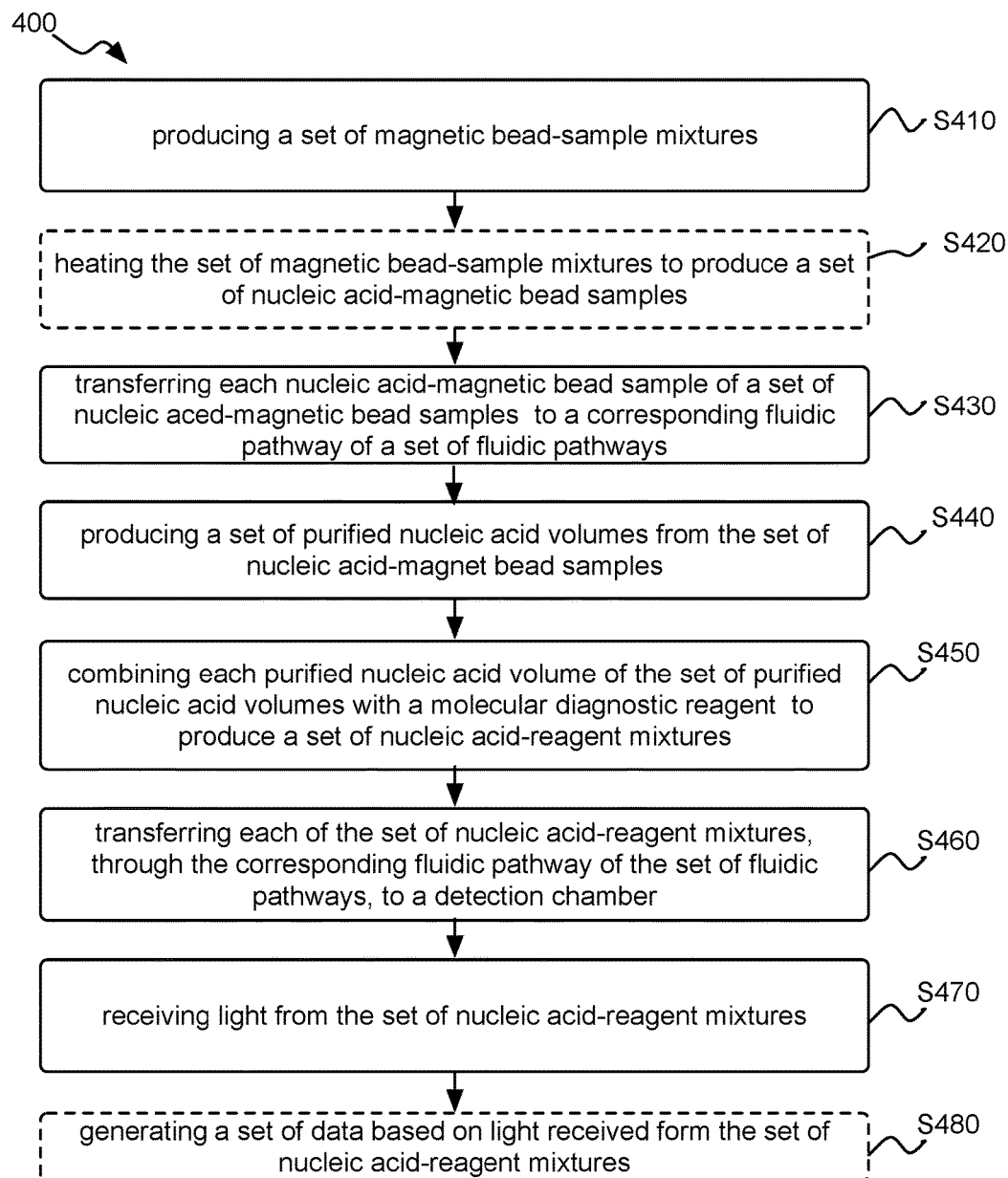


FIGURE 12

US 10,093,963 B2

1

SYSTEM AND METHOD FOR PROCESSING BIOLOGICAL SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 14/229,396, filed 28 Mar. 2014, which is a continuation-in-part of U.S. application Ser. No. 13/766,359, filed 13 Feb. 2013, which claims the benefit of U.S. Provisional Application No. 61/667,606, filed 3 Jul. 2012 and U.S. Provisional Application No. 61/598,240, filed 13 Feb. 2012, all of which are incorporated herein in their entirety by this reference.

TECHNICAL FIELD

This invention relates generally to the molecular diagnostics field, and more specifically to an improved system and method for processing biological samples.

BACKGROUND

Molecular diagnostics is a clinical laboratory discipline that has developed rapidly during the last 25 years. It originated from basic biochemistry and molecular biology research procedures, but now has become an independent discipline focused on routine analysis of nucleic acids (NA), including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for diagnostic use in healthcare and other fields involving analysis of nucleic acids of biological samples. Molecular diagnostic analysis of biological samples can include the detection of one or more nucleic acid materials present in a specimen. The particular analysis performed may be qualitative and/or quantitative. Methods of analysis typically involve isolation, purification, and amplification of nucleic acid materials, and polymerase chain reaction (PCR) is a common technique used to amplify nucleic acids. Often, a nucleic acid sample to be analyzed is obtained in insufficient quantity, quality, and/or purity, hindering a robust implementation of a diagnostic technique. Current sample processing methods and molecular diagnostic techniques are often labor/time intensive, low throughput, and expensive, and systems of analysis are insufficient. Furthermore, methods of isolation, processing, and amplification are specific to certain sample matrices and/or nucleic acid types and not applicable across common biological sample and nucleic acid types.

Due to these and other deficiencies of current molecular diagnostic systems and methods, there is thus a need for and improved system and method to facilitate processing of biological samples. This invention provides such a system and method.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-1B depict an embodiment of a system for processing biological samples;

FIGS. 2-3 depict embodiments of a molecular diagnostic module for processing biological samples;

FIGS. 4A-4B depict configurations of a linear actuator of an embodiment of a molecular diagnostic module;

FIGS. 5A-5D depict elements of an embodiment of a valve actuation subsystem of a molecular diagnostic module;

FIGS. 6A-6B depict configurations of an embodiment of a valve actuation subsystem of a molecular diagnostic module;

2

FIGS. 7A-7C depict elements of an embodiment of a valve actuation subsystem of a molecular diagnostic module;

FIGS. 8A and 8B depict elements of an example of a valve actuation subsystem of a molecular diagnostic module;

FIG. 9 depicts elements of an embodiment of an optical subsystem of a molecular diagnostic module;

FIGS. 10A-10B depict an embodiment of a microfluidic cartridge for processing biological samples;

FIGS. 11A-11C depict an embodiment of a fluid handling system of a system for processing and detecting nucleic acids; and

FIG. 12 depicts an embodiment of a method for processing and detecting nucleic acids.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description of preferred embodiments of the invention is not intended to limit the invention to these preferred embodiments, but rather to enable any person skilled in the art to make and use this invention.

1. System for Processing and Detecting Nucleic Acids

As shown in FIGS. 1A-1B, an embodiment of a system **100** for processing biological samples includes: a molecular diagnostic module **130** comprising a microfluidic cartridge receiving module **140**, heating and cooling subsystem **150**, a magnet **160**, a valve actuation subsystem **170**, and an optical subsystem **280**. Other embodiments of the system **100** can further comprise a microfluidic cartridge **210** configured to facilitate sample processing; a liquid handling system **250** configured to facilitate gas and fluid delivery to different elements of the system **100**; a processor **290** configured to analyze data resulting from a run of the system **100**; and a user interface **295** configured to allow a user to interact with the system **100**. The system **100** can additionally or alternatively include any other suitable elements, as described in U.S. application Ser. No. 13/766,359 entitled "System and Method for Processing and Detecting Nucleic Acids" and filed on 13 Feb. 2013, which is incorporated herein in its entirety by this reference. The system **100** thus functions to receive biological samples containing nucleic acids (i.e., impure nucleic acid samples), separate nucleic acids from the biological samples, and analyze nucleic acid samples according to at least one molecular diagnostic protocol (e.g., PCR). Preferably, the system **100** is a walk-away system by which a user loads a set of biological samples containing nucleic acids, and receives a set of data resulting from a molecular diagnostic protocol without any further sample manipulation by the user. Alternatively, the system **100** facilitates aspects of sample preparation for a molecular diagnostic protocol, with some sample manipulation performed by the user.

In one example workflow of the system **100**, a liquid handling system **250** aspirates a set of biological samples and dispenses the biological samples into a microfluidic cartridge **210**, aligned within a cartridge receiving module **140** of a molecular diagnostic module **130**, and configured to be manipulated by the molecular diagnostic module **130**. A heating and cooling subsystem **150**, a magnet **160**, and a valve actuation subsystem **170** of the molecular diagnostic module **130** then facilitate separation of a set of nucleic acids from the biological samples, as the liquid handling system **250** dispenses wash solutions, release solutions, and/or air at appropriate stages. The liquid handling system **250** then aspirates the set of nucleic acids from the microfluidic cartridge **210** contained within the molecular diagnostic

module **130**, combines the set of nucleic acids with a set of molecular diagnostic reagents, and dispenses the set of nucleic acids combined with the set of molecular diagnostic reagents (i.e., set of nucleic acid-reagent mixtures) into the microfluidic cartridge **210** within the molecular diagnostic module **130**. The heating and cooling subsystem **150**, optical subsystem **180** and valve actuation subsystem **170** of the molecular diagnostic module **130** then facilitate analysis of the set of nucleic acid-reagent mixtures by a processor configured to display information on a user interface.

As stated, the above workflow is just one example workflow of the system **100**. A detailed description of elements of an embodiment of the system **100** is described in sections 1.1-1.4 below.

1.1 System—Molecular Diagnostic Module

As shown in FIG. 1B, an embodiment of the molecular diagnostic module **130** of the system **100** includes a cartridge receiving module **140**, a heating and cooling subsystem **150**, a magnet **160**, a valve actuation subsystem **170**, and an optical subsystem **280**, and functions to manipulate a microfluidic cartridge **210** for processing of a biological sample containing nucleic acids. The molecular diagnostic module **130** is preferably configured to operate in parallel with at least one other molecular diagnostic module **130**, such that multiple microfluidic cartridges **210** containing biological samples may be processed simultaneously. In a first variation, the molecular diagnostic module **130** is configured to be stackable with another molecular diagnostic module **130** in a manner that enables access to a microfluidic cartridge **210** within each molecular diagnostic module **130**. In another variation, the molecular diagnostic module **130** may not be configured to stack with another molecular diagnostic module, such that the molecular diagnostic modules **130** are configured to rest side-by-side on the same plane. Elements of an embodiment of the molecular diagnostic module **130** are further described in sections 1.1.1 to 1.1.4 below.

1.1.1 Molecular Diagnostic Module—Cartridge Receiving Module

As shown in FIGS. 1B, 2, and 3, the cartridge receiving module **140** of the molecular diagnostic module **130** comprises a cartridge platform **141** including a cartridge loading guiderail **142**, a cartridge stop **143**, a magnet receiving slot **144**, and a set of access regions **145**; a linear actuator **146** configured to displace a microfluidic cartridge **210** resting on the cartridge platform **141**; and a set of springs **148** coupled to the cartridge platform **141**. The cartridge receiving module **140** thus functions to receive, align, and compress a microfluidic cartridge **210** for processing of a biological sample according to a molecular diagnostic assay protocol. The cartridge platform **141** is preferably configured to receive a microfluidic cartridge **210** along a cartridge loading guiderail **142** until it reaches a cartridge stop **143**, and be vertically displaced by the linear actuator **146**, which places a biasing force against the set of springs **148** coupled to the cartridge platform **141**. The magnet receiving slot **144** and the set of access regions **145** provide access, by a magnet **160** and a valve actuation subsystem **170**, to the microfluidic cartridge **210**, as the microfluidic cartridge **210** is vertically displaced by the linear actuator **146**.

The cartridge platform **141** includes a cartridge loading guiderail **142**, a cartridge stop **143**, a magnet receiving slot **144**, and a set of access regions **145**, and functions to receive and align a microfluidic cartridge **210**, while providing access to the microfluidic cartridge **210** by a magnet **160** and a valve actuation subsystem **170**. As shown in FIG. 2, an embodiment of the cartridge platform **141** includes a pair of

parallel cartridge loading guiderails **142**, initiating at a pair of inwardly tapering protrusions configured to guide a microfluidic cartridge toward the pair of parallel cartridge loading guiderails **142**, and spanning two short edges of the cartridge platform **141**. The embodiment of the cartridge platform **141** also includes a cartridge stop **143** comprising a vertical tab oriented perpendicular to the cartridge loading guiderails **142**, and spanning a long edge of the cartridge platform. Preferably, the cartridge loading guiderails **142** and the cartridge stop **143** are configured such that a microfluidic cartridge **210** slides between the cartridge loading guiderails **142** and hits the cartridge stop **143** to signal proper alignment. Alternatively, the cartridge loading guiderails **142** and the cartridge stop **143** can be configured such that a microfluidic cartridge slides over or along the cartridge loading guiderails **142**, after which the cartridge stop **143** couples to a portion of the microfluidic cartridge **210** to ensure proper alignment of the microfluidic cartridge. Additional variations of the cartridge loading guiderails **142** and the cartridge stop **143** can be used to enable reception and alignment of a microfluidic cartridge **210** by the molecular diagnostic module **130**.

The embodiment of the cartridge platform **141** shown in FIG. 2 also includes a set of access regions **145**, oriented perpendicular to the parallel cartridge loading guiderails **142** and configured to provide access to a valve actuation subsystem **170**, and a magnet receiving slot **144** located among the set of access regions **145**. Preferably, the magnet receiving slot **144** and the set of access regions **145** substantially span a long dimension of the cartridge platform **141**, as shown in FIG. 2, and are configured to correspond to locations on a microfluidic cartridge **210** requiring a magnetic field and/or valving to enable processing of a biological sample and nucleic acid detection once the microfluidic cartridge **210** has been aligned within the molecular diagnostic module **130**. Thus, alternative configurations of the magnet receiving slot **144** and the set of access regions **145** can accommodate other cartridges with alternative regions requiring magnetic fields and/or valving to enable other protocols. In one alternative embodiment, the magnet receiving slot **144** and the access regions can comprise one continuous void of the cartridge platform **141**, such that the cartridge platform **141** supports a microfluidic cartridge **210** along the periphery of the microfluidic cartridge **210**, but forms a continuous void under a majority of the footprint of the microfluidic cartridge **210**.

The linear actuator **146** functions to linearly displace a microfluidic cartridge **210** resting on the cartridge platform **141**, in order to compress the microfluidic cartridge **210** and position the microfluidic cartridge **210** between a cartridge heater **153** and an optical subsystem **280** on one side of the microfluidic cartridge **210**, and a magnet **160** and detection chamber heaters **157** on another side of the microfluidic cartridge **210**, as described in further detail below. The linear actuator **146** also functions to provide a sufficient counterforce to the valve actuation subsystem **170** such that a microfluidic cartridge **210** within the molecular diagnostic module **130** remains properly situated upon manipulation by the valve actuation subsystem **170**. The linear actuator **146** further functions to move a nozzle **149** coupled to the liquid handling system **250**, in order to couple the liquid handling system **250** to a fluid port **222** of the microfluidic cartridge **210**. In the orientation of the molecular diagnostic module **130** shown in FIG. 1B, the linear actuator **146** is preferably coupled to the cartridge heater **153** and a portion of the optical subsystem **280**, and vertically displaces the cartridge heater **153** and the optical subsystem **280** to

position the cartridge heater **153** and the optical subsystem **280** over the microfluidic cartridge **210**. The vertical displacement also allows the microfluidic cartridge **210** to receive a magnet **160**, which provides a magnetic field to facilitate a subset of a molecular diagnostic protocol, and detection chamber heaters **157**, which allows amplification of nucleic acids for molecular diagnostic protocols requiring heating and cooling of the nucleic acid (e.g. PCR). Preferably, the linear actuator **146** is a scissor jack actuator configured to apply substantially uniform pressure over all occlusion positions of a microfluidic cartridge **210** aligned within the molecular diagnostic module **130**, and to operate in at least two configurations. In a retracted configuration **146a**, as shown in FIG. **4A**, the scissor jack actuator has not linearly displaced the cartridge platform **141**, and in an extended configuration **146b**, as shown in FIG. **4B**, the scissor jack actuator has linearly displaced the microfluidic cartridge **210** to position the microfluidic cartridge **210** between the subsystems **153**, and **180**, and the magnet **160** and detection chamber heaters **157**. Additionally, the extended configuration **146b** of the scissor jack actuator is configured to couple the nozzle **149** to a fluid port **222** of the microfluidic cartridge **210**, such that the liquid handling system **250** can deliver solutions and gases for processing of biological samples. The linear actuator **146** may alternatively be any appropriate linear actuator, such as a hydraulic, pneumatic, or motor-driven linear actuator, configured to linearly displace a microfluidic cartridge within the molecular diagnostic module **130**.

As shown in FIGS. **1B**, **4A**, and **4B**, a set of springs **148** is coupled to the cartridge platform **141** and functions to provide a counteracting force against the linear actuator **146** as the linear actuator **146** displaces a microfluidic cartridge **210** resting on the cartridge platform **141**. The set of springs **148** thus allows the cartridge platform **141** to return to a position that allows the microfluidic cartridge **210** to be loaded and unloaded from the molecular diagnostic module **130** when the linear actuator **146** is in a retracted configuration **146b**, as shown in FIG. **4B**. Similarly, the nozzle **149**, the heating and cooling subsystem **150**, the cartridge heater **153**, and the magnet **160** are preferably coupled to springs, such that springs are positioned between elements **149**, **150**, **153**, and **160**, and substrates that elements **149**, **150**, **153**, and **160** are mounted to. Alternatively an elastomeric material is preferably positioned between elements **149**, **150**, **153**, and **160**, and substrates that elements **149**, **150**, **153**, and **160** are mounted to. The springs and/or elastomeric material function to provide proper functioning and alignment of subsystems of the molecular diagnostic module **130** as the linear actuator **146** is extended or retracted, contributing to reliability and a reduction in stack up tolerance risk. The springs and/or elastomeric material further function to allow more pressure to be applied to occlusion positions of a microfluidic cartridge **210** aligned within the molecular diagnostic module **130**, and an appropriate pressure to be applied to elements **149**, **150**, **153** and **160** of the molecular diagnostic module **130**. Thus, proper contact is maintained between elements **149**, **150**, **153**, and **160**, and a microfluidic cartridge **210** being manipulated by the molecular diagnostic module. These elements are described in further detail below.

1.1.2 Molecular Diagnostic Module—Heating/Cooling Subsystem and Magnet

The heating and cooling subsystem **150** of the molecular diagnostic module **130** comprises a cartridge heater **153**, a fan **155**, and a set of detection chamber heaters **157** and functions to controllably heat portions of a microfluidic

cartridge **210** for processing of a biological sample containing nucleic acids according to a molecular diagnostic protocol. In the orientation of an embodiment of the molecular diagnostic module **130** shown in FIG. **1B**, the cartridge heater **153** is preferably coupled to the linear actuator **146** of the cartridge receiving module **140** and configured to span a central region of a microfluidic cartridge **210** aligned within the molecular diagnostic module **130**, the fan **155** is located at a back wall of the cartridge receiving module **140**, and the set of detection chamber heaters **157** is located inferior to a set of detection chambers of the microfluidic cartridge **210**. In alternative embodiments of the molecular diagnostic module **130**, the heating and cooling subsystem **150** can have any appropriate alternative configuration that provides controlled heating and cooling to a microfluidic cartridge within the molecular diagnostic module **130**.

The cartridge heater **153**, the fan **155**, and the set of detection chamber heaters **157** are preferably those described in U.S. application Ser. No. 13/766,359 entitled “System and Method for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013, which is incorporated herein in its entirety by this reference; however, in other variations, the cartridge heater **153**, the fan **155**, and/or the set of detection chamber heaters **157** can be any other suitable cartridge heater **153**, fan **155**, and/or set of detection chamber heaters **157**.

The magnet **160** of the molecular diagnostic module **130** functions to provide a magnetic field for isolation and extraction of nucleic acids bound to magnetic beads within a microfluidic cartridge **210**, aligned within the molecular diagnostic module **130**. Preferably, the magnet **160** is fixed within the molecular diagnostic module **130**, such that the extended configuration **146b** of the linear actuator **146** allows the magnet **160** to pass through the magnet receiving slot **144** of the cartridge receiving module **140** and into a magnet housing region **218** of the microfluidic cartridge **210**. In an example, the magnet **160** is a rectangular prism-shaped magnet **160** fixed under the cartridge platform **141**, and configured to pass through the cartridge platform **141**, into a magnet housing region **218** located under the heating region of the microfluidic cartridge **210**. Preferably, the magnet **160** is one of two or three magnets lined up in parallel, such that each of the fluidic pathways of a microfluidic cartridge housing the magnets is exposed to two or three times as much magnetic flux, and two to three times as many opportunities to capture magnetic beads. Alternatively, the magnet **160** is a single magnet configured to expose a set of fluidic pathways to a magnetic field. Preferably, the magnet **160** or group of multiple magnets is coupled to a magnet holder within the molecular diagnostic module **130**. Additionally, the magnet holder is preferably composed of an insulating material, such that the magnet holder does not interfere with proper functioning of the cartridge heater **153**. Alternatively, the magnet holder may not be composed of an insulating material.

The magnet **160** is preferably a magnet **160** as described in U.S. application Ser. No. 13/766,359 entitled “System and Method for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013; however, in other variations, the magnet **160** can be any other suitable magnet. Alternative configurations and/or compositions of the magnet **160** may also be appropriate in facilitating isolation and extraction of nucleic acids bound to magnetic beads within the microfluidic cartridge **210**.

1.1.3 Molecular Diagnostic Module—Valve Actuation Subsystem

As shown in FIGS. 5A-5D, the valve actuation subsystem 170 includes an actuation substrate 171 including an array of active regions 172; a set of pins 174, each pin 175 in the set of pins 174 including a base end 176, a distal end 177, and a displacement region 178; a pin housing 180 including a set of cavities 181 that surround and guide displacement of each pin 175 in the set of pins 174; a spring plate 183 including a set of springs 184 coupled to the set of pins 174; and an actuator 187 coupled to at least one of the actuation substrate 171, the pin housing 180, and the spring plate 183, and configured to provide relative displacement between the array of active regions 172 and the set of pins 174. The valve actuation subsystem 170 functions to manipulate a set of occlusion positions of a microfluidic cartridge 210, as described in further detail below, wherein the set of occlusion positions affect sample and/or fluid (e.g., air, reagent, etc.) transfer through fluidic pathways of the microfluidic cartridge 210. As such, the valve actuation subsystem 170 enables portions of the fluidic pathway(s) to be reversibly opened and closed, or maintained in a closed position, by actuation of the set of pins 174. The valve actuation subsystem 170 is preferably situated at a location inferior to that of the cartridge platform 141, as shown in FIG. 1B, such that the set of pins occlude occlusion positions of the microfluidic cartridge 210 in an inferior to superior direction; However, in one alternative variation, the valve actuation subsystem 170 can be situated at a location superior to that of the cartridge platform 141, such that the set of pins occlude occlusion positions of the microfluidic cartridge 210 in a superior to inferior direction. In still other variations, the valve actuation subsystem 170 can be situated relative to the cartridge platform 141 in any other suitable manner.

The actuation substrate 171 includes an array of active regions 172, which function to interact with one or more displacement regions 178 of the set of pins 174, in order to transition pins 175 of the set of pins 174 into extended configurations 175a and/or retracted configurations 175b, as shown in FIGS. 6A and 6B. The array of active regions 172 is preferably configured such that subsets of the set of pins 174 can be placed into specific and repeatable configurations (e.g., an array of extended and retracted configurations), in order to manipulate fluidic pathways of the microfluidic cartridge 210 in a repeatable manner for processing nucleic acids. Preferably, the actuation substrate 171 transforms lateral motion of the actuation substrate 171 into vertical actuation of one or more pins of the set of pins 174, in the orientation shown in FIGS. 5A and 5C; however, in other variations, the valve actuation subsystem 171 can be configured to transform any other suitable motion (e.g., rotational motion, non-lateral motion, etc.) of the actuation substrate 171 into actuation of one or more pins of the set of pins 174.

The array of active regions 172 preferably includes elements that physically contact the set of pins 174 during motion of the actuation substrate 171, thereby directly moving one or more pins 175 of the set of pins 174 into a desired configuration. In one such variation, the array of active regions 172 includes at least one protrusion (e.g., peak) and can additionally or alternatively include at least one recessed area (e.g., valley) that physically enable raising and/or lowering of the pin(s) of the set of pins 174. In one alternative, the array of active regions 172 can include elements that transmit forces to the pin(s) of the set of pins 174, thereby moving the pin(s) into a desired configuration without physical contact. In one such variation, the array of

active regions 172 includes an array of magnetic elements that produce magnetic fields that enable raising and/or lowering of the pin(s) of the set of pins 174. In any of these variations, elements of the array of active regions 172 can be situated at a first surface 179 of the actuation substrate 171, such that motion of the actuation substrate 171 is transformed into actuation of pins contacting the first surface 179 of the actuation substrate. The first surface 179 can be an inferior surface of the actuation substrate 171, in the orientation shown in FIGS. 5A and 5C, or a superior surface of the actuation substrate 171. In other variations, however, the elements of the array of active regions 172 can be situated at multiple surfaces of the actuation substrate 171, and can additionally or alternatively be embedded within the actuation substrate 171 (e.g., as in an embedded array of magnetic elements). Furthermore, the actuation substrate 171 can include combinations of elements that physically contact the set of pins 174 and elements that transmit forces to the set of pins 174 without physical contact.

In a first variation, as shown in FIG. 5C, the actuation substrate 171 comprises a cam card 173 including a set of peaks 185 and valleys 186, and functions to transform linear motion in one plane to vertical motion in another plane. In the first variation, the cam card 173 contacts the displacement regions 178 of pins in a set of pins 172, such that when a peak 185 of the cam card 173 enters alignment with a displacement region 178, the pin is in one configuration, and when a valley 186 of the cam card 173 enters alignment with a displacement region, the pin is in another configuration. Every peak in the set of peaks 185 and valleys 186 is preferably identical in morphology and dimension; however, in variations, the set of peaks 185 and valleys 186 can include one or more peaks having different dimensions and/or morphologies from other peaks in the set of peaks 185 and valleys 186. Similarly, every valley in the set of peaks 185 and valleys 186 is preferably identical in morphology and dimension; however, in variations, the set of peaks 185 and valleys 186 can include one or more valleys having different dimensions and/or morphologies from other valleys in the set of peaks 185 and valleys 186. As such, the set of peaks 185 and valleys 186 can be configured to provide an identical range of motion for every pin 175 in the set of pins 174, or can be configured to provide different ranges of motion for pins of the set of pins 175. In still other variations, peaks and/or valleys of the set of peaks 185 and valleys 186 can be adjustable in dimension in order to provide adjustable ranges of motion or to compensate for wear of peaks and/or valleys.

In the first variation of the actuation substrate 171, the peaks 185 and valleys 186 of the cam card 173 are preferably in a set configuration, as shown in FIG. 5C, such that lateral motion of the cam card 173 to a set position places the set of pins 174 in a specific configuration in a reversible and/or repeatable manner. The set configuration further functions to enable manipulation of normally closed and normally open occlusion positions, in a specific configuration, of a microfluidic cartridge 210 interacting with the valve actuation subsystem 170. As such, lateral movement of the cam card 173 to different positions of a set of positions consistently places subsets of the set of pins 172 into desired configurations to occlude different portions of a fluidic pathway of a microfluidic cartridge 210 in contact with the set of pins 174. Thus, desired portions of a fluidic pathway of the microfluidic cartridge 210 can be selectively occluded and opened to facilitate processing of a biological sample according to any appropriate tissue, cellular, or molecular diagnostic assay protocol.

In the first variation of the actuation substrate **171**, the set of peaks **185** and valleys **186** of the cam card **173** are situated at a first face **179** of the cam card **173**, such that lateral motion of the cam card **173** is transformed into actuation of pins contacting the first face **179** of the cam card **173**. In the first variation, the first face **179** is oriented away from distal ends **177** of the set of pins **174**, as described in further detail below, such that peaks **185** of the cam card **173** are configured to retract pins of the set of pins **174**, and valleys of the cam card **173** are configured to enable extension of pins of the set of pins. In another variation, however, the set of peaks **185** and valleys **186** can be situated at multiple faces of the cam card **173**.

In the first variation, the cam card **173** is configured to be laterally displaced in one coordinate direction within a plane (e.g., by a linear actuator); however in another variation, the cam card **173** is configured to be laterally displaced in only multiple directions within a plane (e.g., by multiple linear actuators, by an x-y linear actuator). In a specific example, the peaks **185** of the cam card **173** are raised 1 mm above the valleys **186** of the cam card **173**. In the specific example, the peaks **185** are identical and have a substantially semicircular cross section with a radius of 1 millimeter, while the valleys **186** are substantially planar and situated in line with the base of each peak **185**. Alternative variations can, however, include any appropriate configurations and geometries of a cam card **173** with peaks **185** and valleys **186**, driven by any appropriate actuator.

In alternative embodiments of the actuation substrate **171**, the actuation substrate **171** can be a cam wheel comprising an array of active regions **172** configured to enable actuation of pins contacting a cylindrical surface, and configured to convert rotary motion to linear (i.e., vertical) motion of the set of pins **174**. The cam wheel can be configured to contact base ends **176** of pins in the set of pins **174**, and can be coupled to a motor shaft and driven by a motor. In other alternative embodiments of the actuation substrate **171**, the actuation substrate **171** can altogether be replaced by a set of cams, each configured to individually rotate about an axis. In these alternative embodiments, rotating subsets of the set of cams raises/lowers corresponding subsets of the set of pins **174**, and occludes specific portions of a of a microfluidic cartridge **210** in contact with the set of pins **174**.

The set of pins **174** is configured to interact with the actuation substrate **171**, and functions to enable selective occlusion and/or opening of fluidic pathways of the microfluidic cartridge **210**, by way of the set of occlusion positions. The pins of the set of pins **174** preferably include cylindrical portions and, in the orientation shown in FIGS. **5A** and **5C**, each pin in the set of pins **174** preferably has a portion defining a circular cross section configured to facilitate sliding within a pin housing **180**. Alternatively, each pin can comprise any appropriate cross-sectional geometry (e.g., rectangular) and/or end shape (e.g., flat or pointed) to facilitate occlusion of a fluidic pathway of a microfluidic cartridge **210**. Preferably, the surface of each pin in the set of pins **174** is composed of a low-friction material to facilitate sliding motions (i.e., in cooperation with an actuation substrate **171** or within a pin housing **180**); however, each pin may alternatively be coated with a lubricant configured to facilitate sliding motions. In a specific example, the valve actuation subsystem **170** comprises 12 sets of pins **174** configured to selectively occlude 12 fluidic pathways of a microfluidic cartridge **210** aligned within the molecular diagnostic module **130**; however, other embodiments can comprise any other suitable number of sets of pins **174**.

Each pin **175** in the set of pins **174** preferably includes a base end **176**, a distal end **177**, and a displacement region **178**. As shown in FIG. **5C**, one or more pins **175** of the set of pins **174** can additionally include a stop region **188** configured to interface with the pin housing **180** in a manner that defines a range of motion for the pin(s) including a stop region **188**. The base end **176** of a pin **175** functions to interact with springs of a spring plate **183**, wherein the springs **184** provide a biasing force against pins of the set of pins **174**, as described in further detail below. As such, the base end **176** can be configured to abut a spring of the spring plate **183**, and can additionally or alternatively be coupled to the spring of the spring plate **183** in any other suitable manner. The distal end **177** of a pin **175** functions to interact with an occlusion position of a fluidic pathway of a microfluidic cartridge **210** aligned with the valve actuation subsystem **170** of an embodiment of the molecular diagnostic module **130**. The base end **176** and/or the distal end **177** of a pin **175** can be cylindrical in morphology, in order to provide an occlusion surface that is substantially circular, ovaloid, or ellipsoidal. However, in some variations, the base end **176** and/or the distal end **177** of pin **175** can be polygonal prismatic (e.g., square prismatic, triangular prismatic) in order to provide an occlusion surface that is polygonal, or amorphous prismatic in morphology. Furthermore, in alternative variations, any suitable portion of a pin **175** can be configured to interact with a spring in order to provide a biasing force against the pin **175**.

The displacement region **178** of a pin **175** functions to interact with one or more active regions of the array of active regions to enable actuation of the pin **175**. Preferably, the displacement region **178** is substantially aligned with the actuation substrate **171**, in order to facilitate interaction between an active region **172** of the actuation substrate **171** and a displacement region **178** of a pin **175**. Additionally, the displacement region **178** preferably mechanically complements one or more active regions **172** of actuation substrate **171**. As such, the displacement region can include a portion that physically contact an active region **172** during motion of the actuation substrate **171**, and in one such variation, the displacement region **178** includes at least one protrusion (e.g., peak) or recessed area (e.g., valley) that physically interacts with the active region to raise or lower the pin **175**. In one alternative, the displacement region **178** can include additionally or alternatively include a portion that responds to forces provided by the active region(s) **172** of the actuation substrate **171**, thereby moving the pin into a desired configuration without physical contact between the pin **175** and an active region. In one such variation, the displacement region can include a magnetic element that responds to magnetic fields produced by active regions of the actuation substrate **171** to raise or lowering the pin. In this variation, the magnetic element of the displacement region **178** can be configured to be drawn toward the magnetic element of an active region **172**, or can be configured to be repelled away from the magnetic element of the active region **172**.

As shown in FIGS. **7A-7C**, the displacement region **178** of a pin **175** is preferably incorporated within the body of the pin **175**, between the base end **176** and the distal end **177** of the pin **175**. In one variation, the displacement region **178** can be incorporated within an opening **189** defined within the body of the pin **175**. However, the displacement region **178** of a pin **175** can additionally or alternatively be biased toward an exterior surface of the pin **175**, defined at least in part at a recessed region of the exterior surface of the pin **175**, or coupled to the pin in any other suitable manner (e.g.,

US 10,093,963 B2

11

using an extension configured to transmit motion from the displacement region to the pin).

In a first specific example, the displacement region **178** includes a semi-cylindrical protrusion defined within a slotted opening **189**, wherein the base of the semi-cylindrical protrusion is situated at the base end of the slotted opening (i.e., toward the base end **176** of the pin **175**). In the first specific example, the actuation substrate **171** thus comprises at least one arm **199** including an active region **172** (e.g., a peak, a valley), as shown in FIG. 7C, wherein the arm **199** is configured to pass through the slotted opening **189** of the pin **175**, and wherein movement of the arm **199** to place the active region **172** into alignment with the protrusion (i.e., the displacement region **178**) results in actuation of the pin **175**. In a variation of the first specific example, a magnetic element proximal a surface of the slotted opening of the displacement region **178** of the pin **175** can be configured to interact with a corresponding magnetic element (i.e., an active region **172**) of an arm **199** of the actuation substrate **171**. In this variation, the magnetic element of the arm **199** can be configured to magnetically repel or draw the magnetic element of the displacement region **178** of the pin **175**, in order to provide actuation of the pin. In other examples, however, the displacement region **178** of a pin can be configured to complement configurations of active regions **172** of the actuation substrate **171** in any other suitable manner.

The stop region **188** is preferably a protruding portion of a pin **175** that interacts with a complementary portion of the pin housing **180**, such that the complementary portion of the pin housing obstructs further motion of the pin **175**. The stop region **188** can comprise a region of the pin **175** that has a larger width or diameter than other regions of the pin **175**, and can additionally or alternatively include a protrusion that limits range of motion of the pin in any other suitable manner. In an example shown in FIG. 5C, a pin **175** includes a stop region **188** located between the base end **176** and the distal end **177** of a pin **175** and defined by a larger diameter than that of the pin housing **180**. In other variations of the specific example, however, the stop region **188** of the pin **175** can be located at any other suitable position along the length of the pin **175**. Furthermore, each pin **175** of the set of pins **174** can include multiple stop regions **188** configured to define ranges of motion for the set of pins.

Each pin **175** in the set of pins **174** preferably operates in an extended configuration **175a** and/or a retracted configuration **175b**. Preferably, each pin **175** of the set of pins **174** is configured to reversibly and repeatably transition between the extended configuration **175a** and the retracted configuration **175b**. However, in alternative variations, one or more pins **175** of the set of pins **174** can be configured to achieve only one of the extended configuration **175a** and the retracted configuration **175b**, and/or a pin **175** of the set of pins **174** can be configured to be locked semi-permanently in a configuration **175a**, **175b** upon entering the configuration **188**, **189**. In the extended configuration **175a**, the distal end **177** of the pin **175** is configured to protrude from an opening of the pin housing **180**, in order to provide an occluding force at a fluidic pathway of a microfluidic cartridge **210** interacting with the valve actuation subsystem **170**. In the retracted configuration **175b**, the distal end **177** of the pin **175** is configured to retract from the opening of the pin housing **180**, in order to remove an occluding force at a fluidic pathway of a microfluidic cartridge **210** interacting with the valve actuation subsystem **170**. Preferably, in relation to variations described above, the extended configuration is activated by translation of the active region (e.g., a

12

peak, a magnet) away from the displacement region, and the retracted configuration is activated by translation of an active region (e.g., a peak, a magnet) of the actuation substrate into alignment with the displacement region. As such, in an example with the orientation shown in FIGS. 6A and 6B, the extended configuration **175a** is activated as a peak of the actuation substrate **171** is moved away from the protrusion of the displacement region **178** of a pin **175**, and the retracted configuration **175b** is activated as a peak of the actuation substrate **171** is moved into alignment with the protrusion of the displacement region **178** (i.e., the peak of the actuation substrate **171** pushes the pin **175** downward by way of the protrusion in the displacement region **178**). The extended and retracted configurations **175a**, **175b** can, however, be activated in any other suitable manner.

The pin housing **180** includes a set of cavities **181**, which function to surround and guide displacement of each pin **175** in the set of pins **174**. As such, the pin housing **180** functions to constrain and guide motion of each pin **175** in the set of pins **174**, as the actuation substrate **171** moves and interacts with the set of pins **174**. In one variation, each pin **175** in the set of pins **174** is surrounded by an individual cavity of the set of cavities **181**; however, in another variation a cavity of the set of cavities **181** can be configured to surround multiple pins in the set of pins **174**. In an example shown in FIGS. 5A-5D, the pin housing **180** is located under the cartridge platform **141**, such that the set of cavities **181** is aligned with the set of access regions **145**, to provide access, by the set of pins **174**, to a microfluidic cartridge **210** aligned on the cartridge platform **141**. In the example, the pin housing **180** thus constrains the set of pins **174**, such that each pin can only move linearly in a vertical direction, and with a set range of motion. Each cavity of the set of cavities **181** preferably has a constricted region (i.e., serving as a pin stop) configured to limit the motion of a pin within a cavity (i.e., by way of the stop region **188** of the pin); however, each cavity of the set of cavities **181** may alternatively not include a constricted region. Preferably, surfaces of the pin housing **180** contacting the set of pins **174** are composed of a low friction material to facilitate sliding of a pin **175** within a cavity of the pin housing **180**; however, surfaces of the pin housing **180** contacting the set of pins **174** may alternatively be coated with a lubricant configured to facilitate sliding motions. Other variations of the pin housing **180** and the set of pins **174** may include no additional provisions to facilitate sliding of a pin **175** within a cavity of the set of cavities **181**.

The spring plate **183** includes a set of springs **184** coupled to the set of pins **174**, and functions to provide biasing forces against the set of pins, in order to bias each pin in the set of pins in a specific direction. The spring plate **183** is preferably situated proximal the base ends **176** of the set of pins **174**; however, the spring plate **183** can alternatively be configured relative to other elements of the valve actuation subsystem **170** in any other suitable manner. A spring of the set of springs **184** preferably functions to provide a counteracting force to restore a pin to a desired configuration (e.g., an extended configuration **175a**, a retracted configuration **175b**). Furthermore, a spring of the set of springs **184** can additionally function to allow sufficient force to be transmitted through the pin **175** to fully occlude a microfluidic channel of a microfluidic cartridge **210**, while preventing forces from being generated that could damage the pin **175**, the microfluidic cartridge **210**, and/or the actuation substrate **171**. Preferably, a spring of the set of springs **184** is configured to abut the base end **176** of a pin **175**, and/or a region substantially proximal the base end **176** of the pin **175** in order to transmit a biasing force to the pin **175**. However,

a spring of the set of springs **184** can additionally or alternatively be configured to couple to any other suitable portion of a pin **175**. Furthermore, the set of springs **184** can be configured to bias every pin of the set of pins **174** in the same direction with identical magnitudes of force; however, in other variations, the set of springs **185** can be configured to bias different pins of the set of pins **175** in different directions, and/or with different magnitudes of force.

In a first variation, the set of springs **184** is configured to bias every pin **175** of the set of pins **174** toward an extended configuration **175a**, such that when an active region (e.g., a peak, a magnet) of the actuation substrate **171** substantially enters alignment with a displacement region **178** of a pin **175**, the pin **175** is transitioned into a retracted configuration **175b** and the spring contacting the pin is compressed (e.g., further compressed, transitioned from a neutral state to a state of compression). Then, in the first variation, when the active region of the actuation substrate **171** is moved out of alignment with the displacement region **178**, the pin **175** is restored to an extended state. In other variations, however, the spring(s) of the set of springs **184** can be configured to bias the pin(s) of the set of pins **174** toward a retracted configuration **175b**, such that alignment of the active region(s) of the actuation substrate **171** with the displacement region(s) **178** of the pin(s) transitions the pin(s) into an extended configuration **175a**. In still other variations, the springs can be configured to bias the pins in any other suitable manner.

The actuator **187** is coupled to at least one of the actuation substrate **171**, the pin housing **180**, and the spring plate **183**, and functions to provide relative displacement between the array of active regions **172** and the set of pins **174**, thus transforming motion of the actuation substrate **171** into motion of subsets of the set of pins **174**. The actuator **187** is preferably a linear actuator; however, the actuator **187** can additionally or alternatively comprise any other suitable actuator. Preferably, the actuator **187** is coupled to the actuation substrate **171** with the set of pins **174**, the spring housing **180**, and the spring plate **183** substantially stationary, such that actuation of the actuator **187** manipulates motion of the set of pins **174** in order to occlude pathways of the microfluidic cartridge **210**. In one such variation, as shown in FIGS. **5C** and **5D**, the actuator can be coupled to one end of the actuation substrate **171** (e.g., using a set of coupling points defined within the actuation substrate **171**); however, in other variations, the actuator **187** can be coupled to any other suitable portion of the actuation substrate **171**. Alternatively, the actuator **187** can be configured to move the set of pins **174**, within the pin housing **180**, relative to the actuation substrate **171**, in order to occlude pathways of the microfluidic cartridge **210**. In still other variations, the actuator **187** can be coupled to any other suitable portion of the valve actuation subsystem **170**.

In one specific example of the valve actuation subsystem **170** shown in FIGS. **5A-5D**, **7A-7C**, and **8A-8B**, the set of pins **174** and the pin housing **180** are located directly under the microfluidic cartridge **210**, after the microfluidic cartridge **210** has been aligned within the molecular diagnostic module **130**, such that the set of pins can access the microfluidic cartridge **210** through the access regions **145** of the cartridge platform **141**. The actuation substrate **171** in the specific example is situated amongst the set of pins **174**, between base ends and distal ends of the set of pins **174**, and comprises a set of arms including the active regions **172** (i.e., peaks and valleys) defined at a bottom face of the arms **199** of the actuation substrate, in the orientation shown in FIG. **5C**. In the specific example, peaks **185** of the actuation

substrate **171** are configured to push pins downward to a retracted configuration **189** by way of semi-cylindrical protrusions defined within slotted openings **189** of the pins **175**, and valleys **186** of the actuation substrate **171** are configured to restore pins **175** of an extended configuration **188**. In the specific example, The actuation substrate **171** includes four parallel arms **199** configured to manipulate eight occlusion positions of a microfluidic cartridge **210**, with each arm **199** configured to pass through slotted openings in two pins **175**, in order to manipulate two occlusion positions of the microfluidic cartridge **210**. The four parallel arms **199** include a first arm including two peaks and two valleys, in alternation, configured to manipulate two normally open occlusion positions, a second arm including two valleys, configured to manipulate two normally closed occlusion positions, a third arm including two peaks and two valleys, in alternation, configured to manipulate two normally open occlusion positions, and a fourth arm including a peak and two valleys, configured to manipulate one normally open occlusion position and one normally closed occlusion position. Normally open and normally closed occlusion positions are further described in U.S. application Ser. No. 13/765,996 filed on 13 Feb. 2013 and entitled "Microfluidic Cartridge for Processing and Detecting Nucleic Acids", which is incorporated herein in its entirety by this reference. In the specific example, each arm has a width of ~1.22 millimeters, and is spaced apart from other arms by a gap of ~1 millimeter. The actuation substrate **171** in the specific example further has a maximum width of ~8.74 millimeters and a length of 13.74 centimeters, with peaks having heights 1 millimeter and valleys having depths of 1 millimeter.

In the specific example, the actuation substrate **171** is coupled to an actuator **187** at an end opposite the arms **199** of the actuation substrate **171**, by way of three coupling points, as shown in FIG. **5D**. The actuator **187** is configured to laterally displace the actuation substrate **171** to vertically displace one or more pins **175** of the set of pins **174**. The actuation substrate in the specific example travels on a low friction surface configured to facilitate lateral displacement of the actuation substrate **171**; however, in other variations, the actuation substrate **171** can additionally or alternatively be configured to travel through any other suitable environment having low friction (e.g., air, lubricated surface, surface of ball bearings, etc.) in order to facilitate actuation of the actuation substrate **171**.

While the system **100** preferably includes an embodiment, variation, or specific example of the valve actuation subsystem **170** described above, the system can alternatively or additionally include any other suitable valve actuation subsystem **170**, such as a valve actuation subsystem described in U.S. application Ser. No. 13/766,359 entitled "System and Method for Processing and Detecting Nucleic Acids" and filed on 13 Feb. 2013.

1.1.4 Molecular Diagnostic Module—Optical Subsystem

As shown in FIGS. **1B** and **8**, the optical subsystem **280** of the molecular diagnostic module **130** comprises a set of light emitting elements **281**, a set of excitation filters **282** configured to transmit light from the set of light emitting elements **281**, a set of dichroic mirrors **283** configured to reflect light from the set of excitation filters **282** toward a set of apertures **285** configured to transmit light toward a set of nucleic acid samples, a set of emission filters **286** configured to receive and transmit light emitted by the set of nucleic acid samples, and a set of photodetectors **287** configured to facilitate analysis of light received through the set of emission filters **286**. The optical subsystem **280** can further comprise a set of lenses **284** configured to focus light onto

the set of nucleic acid samples. The optical subsystem 280 thus functions to transmit light at excitation wavelengths toward a set of nucleic acid samples and to receive light at emission wavelengths from a set of nucleic acid samples. Preferably, the optical subsystem 280 is coupled to an optical subsystem actuator 288 configured to laterally displace and align the optical subsystem 280 relative to the set of nucleic acid samples, and is further coupled to a linear actuator 146 of the cartridge receiving module 140 to position the optical subsystem 280 closer to the set of nucleic acid samples. Alternatively, the optical subsystem 280 may not be coupled to a linear actuator 146 of the cartridge receiving module 140, and may only be configured to translate laterally in one direction. In a specific example, the optical subsystem 280 is located within the molecular diagnostic module 130 and coupled to the linear actuator 146 of the cartridge receiving module 140, such that, in the extended configuration 146b of the linear actuator 146, the optical subsystem 280 can be positioned closer to a microfluidic cartridge 210 aligned within the molecular diagnostic module. Conversely in the specific example, the optical subsystem 280 is positioned away from the microfluidic cartridge 210 in the retracted configuration 146a of the linear actuator 146. In the specific example, the optical subsystem 280 is further coupled to an optical subsystem actuator 288 configured to laterally displace the optical subsystem 280 relative to the microfluidic cartridge 210, such that the optical subsystem 280 can be aligned with a set of detection chambers of the microfluidic cartridge 210.

The optical subsystem 280 is preferably an optical subsystem 280 as described in U.S. application Ser. No. 13/766,359 entitled "System and Method for Processing and Detecting Nucleic Acids" and filed on 13 Feb. 2013; however, in other variations, the optical subsystem 180 can additionally or alternatively include any other suitable optical subsystem elements configured to transmit excitation wavelengths of light to samples, and/or receive emission wavelengths of light from the samples.

1.2 System—Microfluidic Cartridge

The microfluidic cartridge 210 functions to receive a set of magnetic bead-samples, facilitate separation of nucleic acids from the set of magnetic bead-samples, receive a set of nucleic acid-reagent samples, and facilitate analysis of nucleic acids from the set of nucleic acid-reagent samples. In one embodiment, as shown in FIGS. 9A and 9B, the microfluidic cartridge 210 comprises a top layer 211 including a set of sample port-reagent port pairs 212 and a set of detection chambers 213; an intermediate substrate 214, coupled to the top layer 211 and partially separated from the top layer 211 by a film layer 215, configured to form a waste chamber 216; an elastomeric layer 217 partially situated on the intermediate substrate 214; a magnet housing region 218 accessible by a magnet 160 providing a magnetic field; and a set of fluidic pathways 219, each formed by at least a portion of the top layer 211, a portion of the film layer 215, and a portion of the elastomeric layer 217. In the embodiment, the microfluidic cartridge 10 further comprises a bottom layer 221 coupled to the intermediate substrate 214 and configured to seal the waste chamber 216. Furthermore, in the embodiment, the top layer 211 of the microfluidic cartridge 210 further comprises a shared fluid port 222, a vent region 223, and a heating region 224, such that each fluidic pathway 220 in the set of fluidic pathways 219 is fluidically coupled to a sample port-reagent port pair 229, the shared fluid port 222, the waste chamber 216, and a detection chamber 225, comprises a turnabout portion configured to pass through the heating region 224 and the

magnetic field, and is configured to pass through the vent region 223 upstream of the detection chamber 225. Each fluidic pathway 220 thus functions to receive and facilitate processing of a sample fluid containing nucleic acids as it passes through different portions of the fluidic pathway 220.

The microfluidic cartridge 210 is preferably configured to be received and manipulated by the molecular diagnostic module 130, such that the cartridge receiving module 140 of the molecular diagnostic module 130 receives and aligns the microfluidic cartridge 210 within the molecular diagnostic module 130, the heating and cooling subsystem 150 of the molecular diagnostic module 130 is configured to transfer heat to the heating region 224 of the microfluidic cartridge 210, and the magnet 160 of the molecular diagnostic module 130 is configured to be received by the magnet housing region 218 of the microfluidic cartridge 210 to provide a magnetic field for separation of nucleic acids. Additionally, the shared fluid port 222 of the microfluidic cartridge 210 is configured to couple to a nozzle 149 coupled to the linear actuator 146 of the cartridge receiving module 140, such that the liquid handling system 250 can deliver fluids and gases through the shared fluid port 222. The elastomeric layer 217 of the microfluidic cartridge 210 is also preferably configured to be occluded at a set of occlusion positions 226 by the valve actuation subsystem 170 of the molecular diagnostic module, in order to occlude portions of a fluidic pathway 220 of the microfluidic cartridge 210 for processing of a set of biological samples. The optical subsystem 180 of the molecular diagnostic module 130 is further configured to align with the set of detection chambers 213 of the microfluidic cartridge 210, to facilitate analysis of a set of nucleic acid samples. The microfluidic cartridge 210 is preferably the microfluidic cartridge 210 described in U.S. application Ser. No. 13/765,996 and filed on 13 Feb. 2013, which is incorporated in its entirety by this reference, but can alternatively be any appropriate cartridge or substrate configured to receive and process a set of samples containing nucleic acids.

1.3 System—Liquid Handling System

The liquid handling system 250 of the system 100 includes a liquid handling arm 255 and a syringe pump 265, as shown in FIGS. 10A-10C and functions to deliver biological samples, reagents, and gases to elements of the system 100. As described in Section 1, an embodiment of the liquid handling system 250 is configured to aspirate a set of biological samples containing nucleic acids (i.e., impure nucleic acid samples), and dispense the set of biological samples into microfluidic cartridge 210 located in a molecular diagnostic module 130. The embodiment of the liquid handling system 100 is further configured to facilitate separation of a set of nucleic acids from the magnetic bead-samples, by dispensing a wash solution, a release solution, and/or air into the molecular diagnostic module 130, by the nozzle 149 coupled to the linear actuator 146, at appropriate stages, aspirate the set of nucleic acids from the molecular diagnostic module 130, combine the set of nucleic acids with a set of molecular diagnostic reagents, and dispense the set of nucleic acids combined with the set of molecular diagnostic reagents (i.e., set of nucleic acid-reagent mixtures) into the molecular diagnostic module 130 for further processing and analysis. Other embodiments of the liquid handling system 250 can be configured to perform alternative molecular diagnostic assay protocols and/or dispense and aspirate alternative fluids into and from other elements supporting a molecular diagnostic protocol.

The liquid handling system 250 is preferably an embodiment of the liquid handling system described in U.S. appli-

cation Ser. No. 13/766,359 entitled “System and Method for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013; however, the liquid handling system **250** can additionally or alternatively include any other suitable elements configured to facilitate delivery of biological samples, reagents, and gases to elements of the system **100**.

1.4 System—Additional Elements

The system **100** can further comprise a controller coupled to at least one of the molecular diagnostic module **130** and the liquid handling system **250**, and functions to facilitate automation of the system **100**. In a variation wherein the controller is coupled to the molecular diagnostic module **130**, the controller preferably functions to automate reception of a microfluidic cartridge, heating of biological samples within the molecular diagnostic module **130** and the detection chambers **213**, occlusion of fluidic pathways **220** by the valve actuation subsystem **170**, and analysis of a set of nucleic acid-reagent mixtures by the optical subsystem **280**. In a variation wherein the controller is coupled to the liquid handling system **250**, the controller preferably functions to automate aspiration, transfer, and delivery of fluids and/or gases to different elements of the system **100**. Other variations of a controller can function to automate handling, transfer, and/or storage of other elements of the system **100**, using a robotic arm or gantry or any other suitable element. Alternative combinations of the above variations can involve a single controller, or multiple controllers configured to perform all or a subset of the functions described above.

The system **100** can also further comprise a processor **290**, which functions to receive and process data received from the optical subsystem **280** of the molecular diagnostic module **130**. Preferably, the processor **290** is coupled to a user interface **295**, which functions to display processed and/or unprocessed data produced by the system **100**, settings of the system **100**, information obtained from a tag reader, or any other appropriate information. Alternatively, the processor **290** is not coupled to a user interface **295**, but comprises a connection configured to facilitate transfer of processed and/or unprocessed data produced by the system **100**, settings of the system **100**, or any other appropriate information to a device external to the system **100**.

The system **100** can further comprise any other suitable element(s) as described in U.S. application Ser. No. 13/766,359 entitled “System and Method for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013, or any other suitable element to facilitate reception or processing of biological samples. As a person skilled in the art will recognize from the previous detailed description and from the figures and claims, modifications and changes can be made the described embodiments of the system **100** without departing from the scope of the system **100**.

2. Method for Processing and Detecting Nucleic Acids

As shown in FIG. **11**, an embodiment of a method **400** for processing and detecting nucleic acids from a set of biological samples comprises: producing a set of magnetic bead-sample mixtures from the set of biological samples **S410**; heating the set of magnetic bead-sample mixtures to produce a set of nucleic acid-magnetic bead samples **S420**; transferring each nucleic acid-magnetic bead sample of the set of nucleic acid-magnetic bead samples to a corresponding fluidic pathway of a set of fluidic pathways **S430**; producing a set of nucleic acid volumes from the set of nucleic acid-magnetic bead samples **S440**; combining each nucleic acid volume of the set of nucleic acid volumes with a molecular diagnostic reagent of a set of molecular diagnostic reagents to produce a set of nucleic acid-reagent

mixtures **S450**; transferring each of the set of nucleic acid-reagent mixtures, through the corresponding fluidic pathway of the set of fluidic pathways, to a detection chamber of a set of detection chambers **S460**; and receiving light from the set of nucleic acid-reagent mixtures **S470**. The method **400** can further include generating a set of data based on light received from the set of nucleic acid-reagent mixtures **S480**. The method **400** functions to isolate and extract a set of nucleic acid volumes from a biological sample, and to facilitate analysis of the nucleic acid volumes according to at least one molecular diagnostic protocol.

The method **400** is preferably implemented at least in part at an embodiment of the system **100** described in Section 1 above; however, the method **400** can additionally or alternatively be implemented at any other suitable system configured to process and detect nucleic acids from a set of biological samples. Preferably, the method **400** is implemented, at least in part, as described in U.S. application Ser. No. 13/766,377 entitled “System and Method for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013, and U.S. application Ser. No. 14/060,214 entitled “Method and Materials for Isolation of Nucleic Acid Materials” and filed on 22 Oct. 2013, which are both incorporated herein in their entirety by this reference; however, the method **400** can additionally or alternatively be implemented in any other suitable manner.

Embodiments of the method **400** and variations thereof can be embodied and/or implemented at least in part by a machine configured to receive a computer-readable medium storing computer-readable instructions. The instructions are preferably executed by computer-executable components preferably integrated with the system **100** and one or more portions of the processor **273** and/or the controller **272**. The computer-readable medium can be stored on any suitable computer-readable media such as RAMs, ROMs, flash memory, EEPROMs, optical devices (CD or DVD), hard drives, floppy drives, or any suitable device. The computer-executable component is preferably a general or application specific processor, but any suitable dedicated hardware or hardware/firmware combination device can alternatively or additionally execute the instructions.

The FIGURES illustrate the architecture, functionality and operation of possible implementations of systems, methods and computer program products according to preferred embodiments, example configurations, and variations thereof. In this regard, each block in the flowchart or block diagrams may represent a module, segment, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that, in some alternative implementations, the functions noted in the block can occur out of the order noted in the FIGURES. For example, two blocks shown in succession may, in fact, be executed substantially concurrently, or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved. It will also be noted that each block of the block diagrams and/or flowchart illustration, and combinations of blocks in the block diagrams and/or flowchart illustration, can be implemented by special purpose hardware-based systems that perform the specified functions or acts, or combinations of special purpose hardware and computer instructions.

As a person skilled in the art will recognize from the previous detailed description and from the figures and claims, modifications and changes can be made to the preferred embodiments of the invention without departing from the scope of this invention defined in the following claims.

US 10,093,963 B2

19

We claim:

1. A system for processing and detecting nucleic acids using a cartridge with a fluidic pathway, the system comprising:

- a cartridge platform configured to receive the cartridge, 5 the cartridge platform comprising an access region configured to align with the fluidic pathway;
- a valve actuation subsystem comprising:
 - a pin comprising a first end, a second end, and a displacement region, the pin actuatable along a displacement axis extending through the first end, the second end, and the access region;
 - a spring, coupled to the displacement region of the pin, that biases the pin along the displacement axis; and 10 an actuation substrate comprising a groove and a protrusion, the actuation substrate translatable along an actuation axis perpendicular to the displacement axis;

wherein the system is operable between:

- a closed configuration, wherein the groove is aligned with the displacement region and the first end of the pin extends through the access region; and
- an open configuration, wherein the protrusion is aligned with the displacement region and the first end of the pin is displaced away from the access region. 25

2. The system of claim 1, wherein the cartridge comprises a set of fluidic pathways, wherein the fluidic pathway is one of the set of fluidic pathways, the system further comprising a magnet, arranged inferior the cartridge platform, that applies a magnetic field spanning at least three fluidic pathways of the set of fluidic pathways. 30

3. The system of claim 1, further comprising an optical subsystem, arranged superior the cartridge platform, that comprises a set of units, wherein each unit includes an excitation filter, an emission filter, a photodetector aligned with the emission filter, and a dichroic mirror. 35

4. The system of claim 1, further comprising a linear actuator, mounted to the cartridge platform, that actuates the cartridge platform along the displacement axis. 40

5. The system of claim 1, further comprising the cartridge, wherein the cartridge includes a set of sample port-reagent port pairs, a fluid port, a set of detection chambers, a waste chamber, and a set of fluidic pathways, wherein the fluidic pathway is one of the set of fluidic pathways, wherein each fluidic pathway is coupled to a sample port-reagent port pair of the set of sample port-reagent port pairs, the fluid port, and a detection chamber of the set of detection chambers, wherein the fluidic pathway is occluded by the pin in the closed configuration. 45

6. The system of claim 1, wherein the valve actuation subsystem further comprises a pin housing surrounding the pin, the pin housing comprising a stop region, proximal the displacement region, that halts pin displacement toward the access region along the displacement axis. 50

7. The system of claim 1, wherein the valve actuation subsystem further comprises a substrate actuator that displaces the actuation substrate along the actuation axis between a first substrate position and a second substrate position along the actuation axis, wherein the actuation substrate is in the first substrate position when the system is in the closed configuration, and is in the second substrate position when the system is in the open configuration. 60

8. The system of claim 1, wherein the displacement region of the pin is arranged proximal the second end, between the first end and second end, and comprises a slot defined along a length of the pin. 65

20

9. The system of claim 8, wherein the actuation substrate passes through the slot, wherein the groove and the protrusion are defined along a face of the actuation substrate parallel the actuation axis.

10. The system of claim 9:

- wherein the spring is mounted to the second end of the pin and biases the pin toward the access region;
- wherein the slot further comprises a pin protrusion extending from an end of the slot proximal the second end; and
- wherein the face of the actuation substrate is proximal the pin protrusion.

11. The system of claim 10, wherein the protrusion and the pin protrusion each comprise a convex profile.

12. A system for processing a biological sample with a cartridge comprising a set of fluidic pathways, each fluidic pathway comprising an occlusion region, the system comprising:

- a pin comprising a first end and a second end, the pin actuatable along a displacement axis between a first pin position and a second pin position, wherein the first end of the pin occludes the occlusion region of a fluidic pathway of the cartridge in the first pin position, and the first end of the pin does not occlude the access region of the fluidic pathway of the cartridge in the second pin position;

an actuation substrate comprising a first substrate feature and a second substrate feature, the actuation substrate actuatable between a first substrate position and a second substrate position; and

- a pin-substrate interface aligning and energetically coupling at least one of the first substrate feature and the second substrate feature with the second end of the pin; wherein the system is operable between:

- a closed state, wherein the actuation substrate is in the first substrate position, the first substrate feature is aligned with the second end of the pin at the pin-substrate interface, and the pin is in the first pin position; and

- an open state, wherein the actuation substrate is in the second substrate position, the second substrate feature aligned with the second end of the pin at the pin-substrate interface, and the pin is in the second pin position.

13. The system of claim 12, wherein the actuation substrate is actuatable between the first substrate position and the second substrate position along an actuation axis perpendicular to the displacement axis, the system further comprising an actuator coupled to the actuation substrate that actuates the actuation substrate along the actuation axis. 50

14. The system of claim 12, wherein the first substrate feature applies one of a neutral or negative force to the pin at the pin-substrate interface, and the second substrate feature applies a positive force to the pin at the pin-substrate interface. 55

15. The system of claim 14, wherein the first substrate feature comprises a groove, and the second substrate feature comprises a protrusion.

16. The system of claim 15, wherein the pin-substrate interface comprises a spring, coupled to the second end of the pin, that applies a spring force opposing the positive force and biases the pin towards the first pin position along the displacement axis.

17. The system of claim 16, wherein the pin-substrate interface further comprises a slot, defined by the pin, proximal the second end.

18. The system of claim 17, wherein the actuation substrate is actuatable between the first substrate position and the second substrate position along an actuation axis perpendicular to the displacement axis, wherein the actuation substrate extends through the slot parallel the actuation axis, 5 wherein the groove and the protrusion are defined along a face of the actuation substrate.

19. The system of claim 18, wherein the groove and protrusion are defined along an inferior face of the actuation substrate; 10 wherein the slot comprises a pin protrusion at an inferior end of the slot; and wherein the spring is mounted to the second end of the pin, inferior the slot.

20. The system of claim 12, further comprising: 15 a cartridge platform configured to receive the cartridge, wherein the valve actuation subsystem is arranged inferior the cartridge platform; a magnet, arranged inferior the cartridge platform, that applies a magnetic field spanning at least three fluidic 20 pathways of the set of fluidic pathways; an optical subsystem, arranged superior the cartridge platform, that comprises an excitation filter, an emission filter, a photodetector aligned with the emission filter, and a dichroic mirror optically connected to the 25 emission filter, cartridge, and the photodetector; and a linear actuator, mounted to the cartridge platform, that actuates the cartridge platform along the displacement axis.

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EXHIBIT 50



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(12) **United States Patent**
Brahmasandra et al.

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(45) **Date of Patent:** ***Mar. 12, 2019**

(54) **THERMOCYCLING SYSTEM AND MANUFACTURING METHOD**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 92 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/363,107**

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(Continued)

(51) **Int. Cl.**
B01L 3/00 (2006.01)
H05K 1/00 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC **B01L 7/52** (2013.01); **H05K 1/18** (2013.01); **H05K 3/30** (2013.01); **H05K 3/4644** (2013.01);
(Continued)

(58) **Field of Classification Search**

CPC B01L 7/525; B01L 2300/1822; B01L 2300/1827; B01L 7/52; B01L 3/502723
See application file for complete search history.

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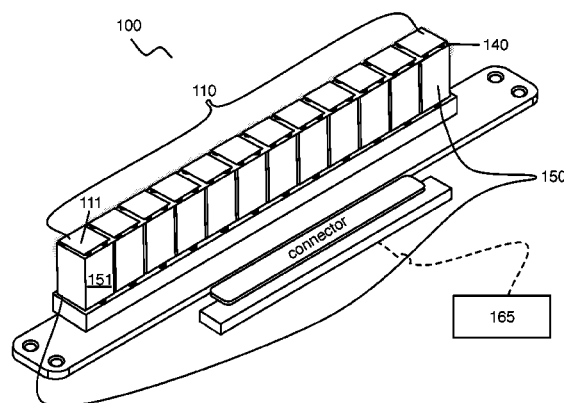
Primary Examiner — Nathan A Bowers

(74) *Attorney, Agent, or Firm* — Jeffrey Schox

(57) **ABSTRACT**

A system for thermocycling biological samples within detection chambers comprising: a set of heater-sensor dies, each heater-sensor die comprising a heating surface configured to interface with a detection chamber and a second surface, inferior to the heating surface, including a first connection point; an electronics substrate, comprising a first substrate surface coupled to the second surface of each heater-sensor die, an aperture providing access through the electronics substrate to at least one heater-sensor die, and a second substrate surface inferior to the first substrate surface, wherein the electronics substrate comprises a set of substrate connection points at least at one of the first substrate surface, an aperture surface defined within the aperture, and the second substrate surface, and wherein the electronics substrate is configured to couple heating elements and sensing elements of the set of heater-sensor dies to a controller; and a set of wire bonds, including a wire bond coupled between the first connection point of at least one of the set of heater-sensor dies and one of the set of substrate connection points.

12 Claims, 10 Drawing Sheets



US 10,226,771 B2

Page 2

Related U.S. Application Data

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H05K 1/18 (2006.01)

H05K 3/30 (2006.01)

H05K 3/46 (2006.01)

(52) **U.S. Cl.**

CPC *B01L 2200/12* (2013.01); *B01L 2200/147*
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2300/123 (2013.01); *B01L 2300/168*
(2013.01); *B01L 2300/1827* (2013.01); *H05K*
2201/10151 (2013.01); *Y10T 29/49083*
(2015.01)

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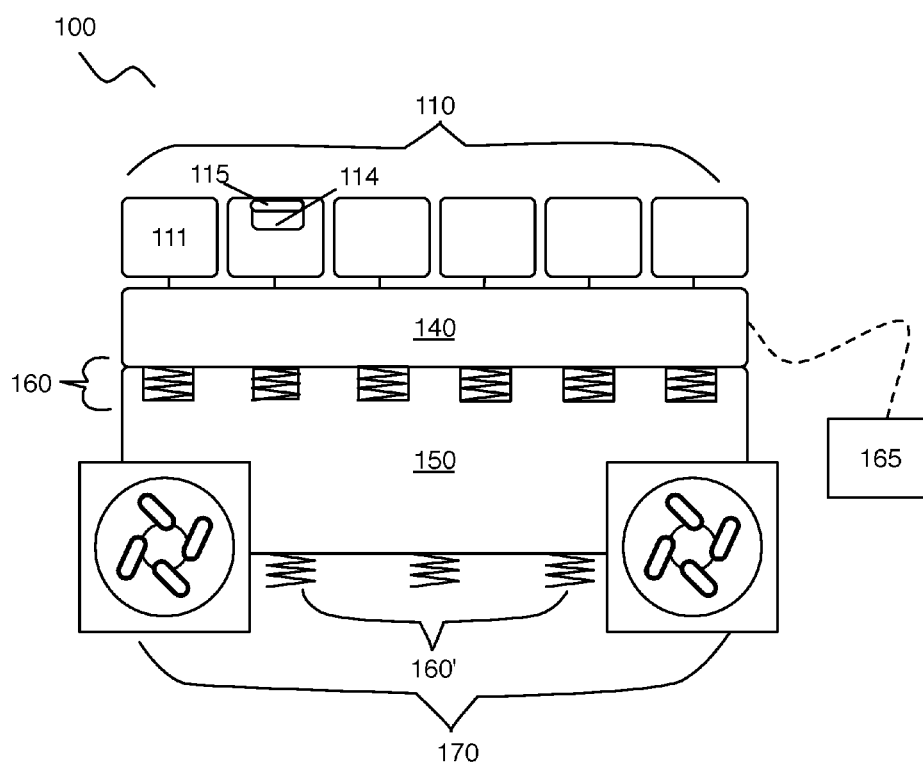


FIGURE 1A

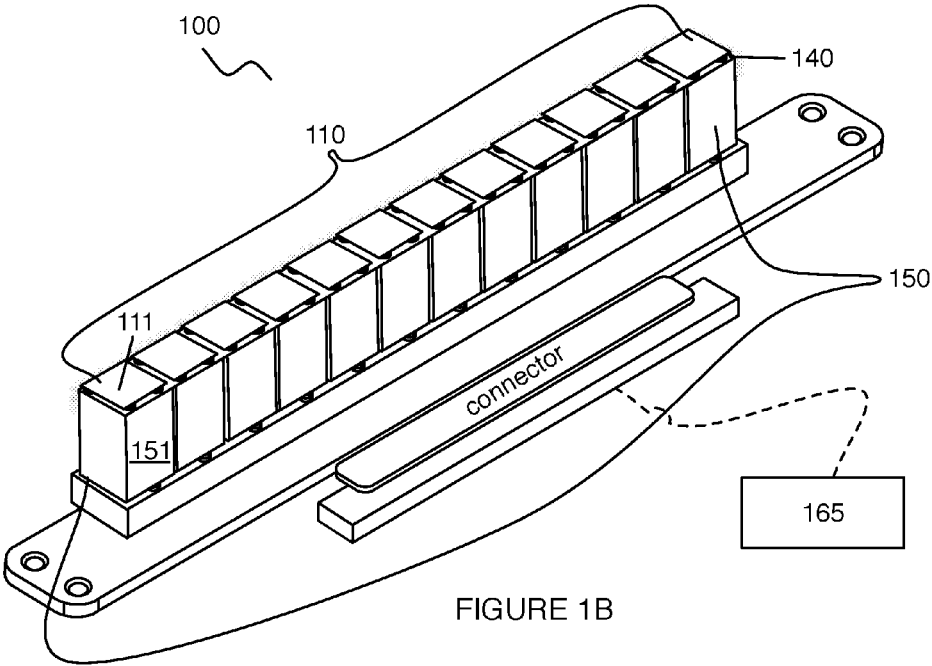


FIGURE 2

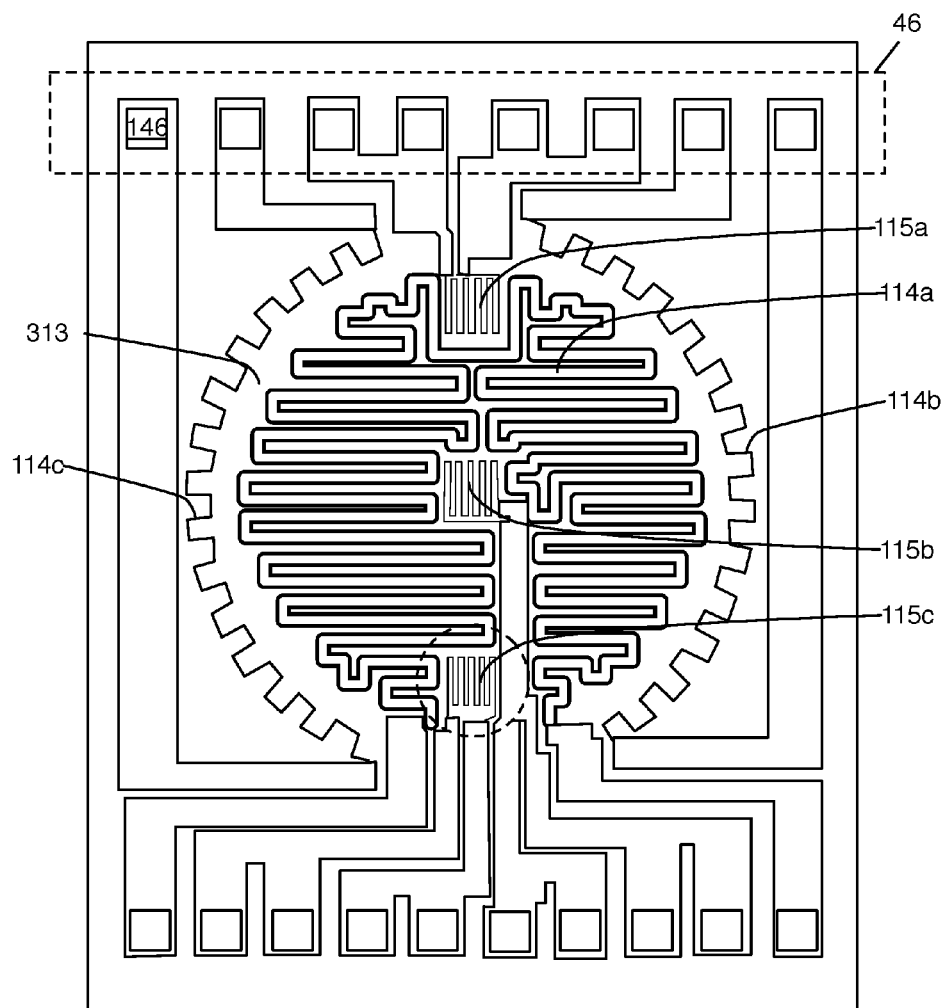


FIGURE 3

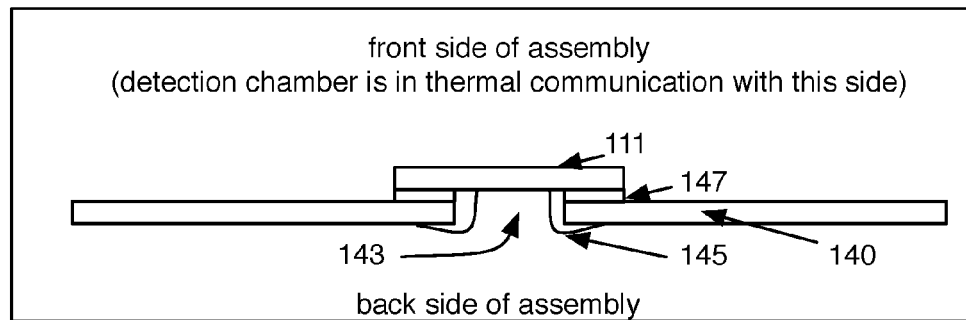
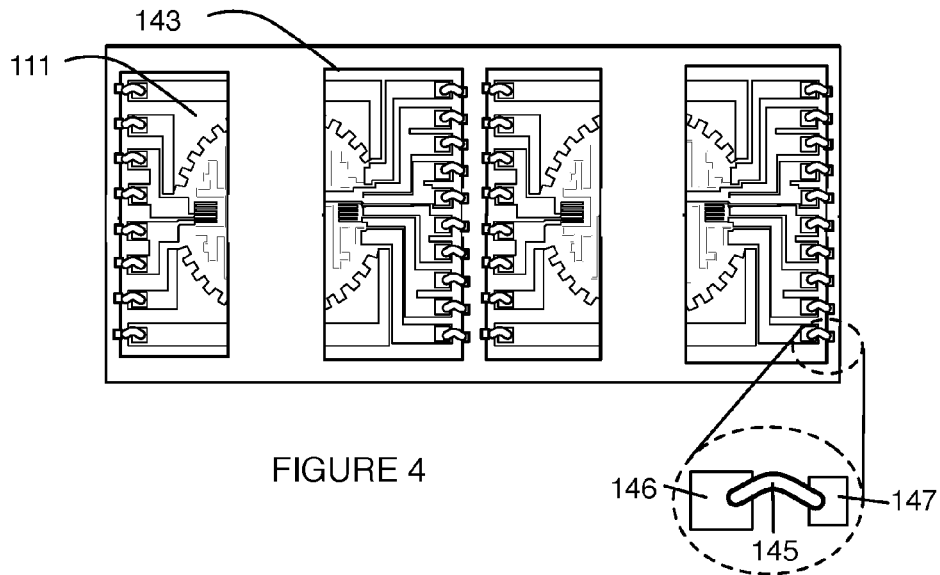


FIGURE 5A

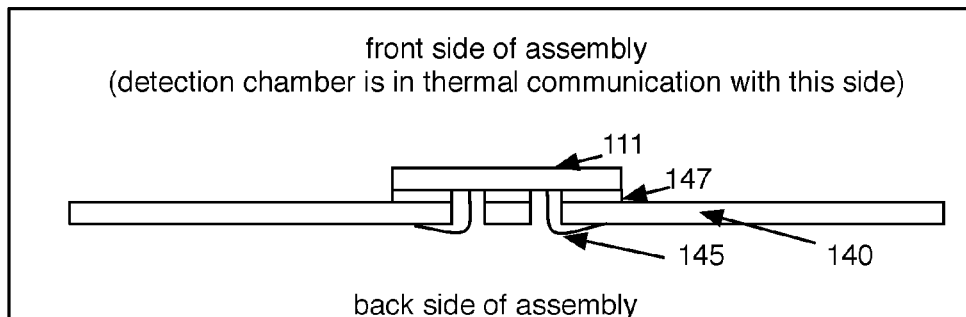


FIGURE 5B

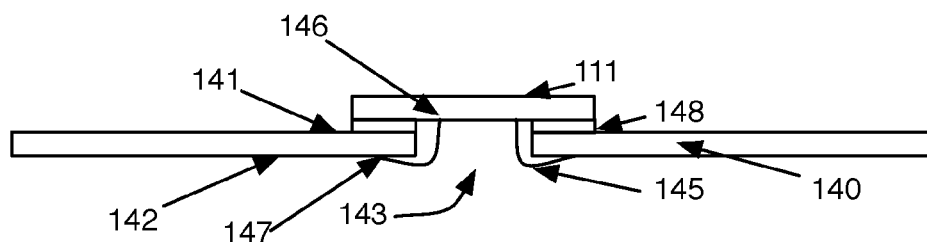


FIGURE 6A

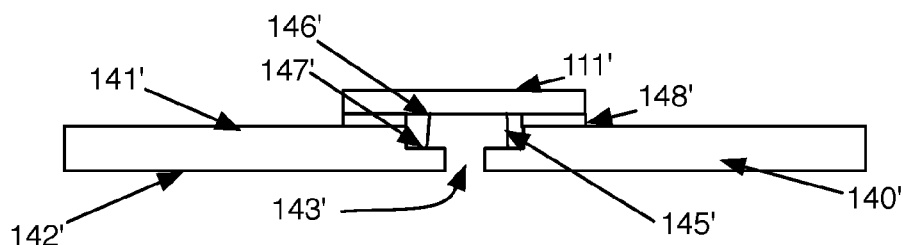


FIGURE 6B

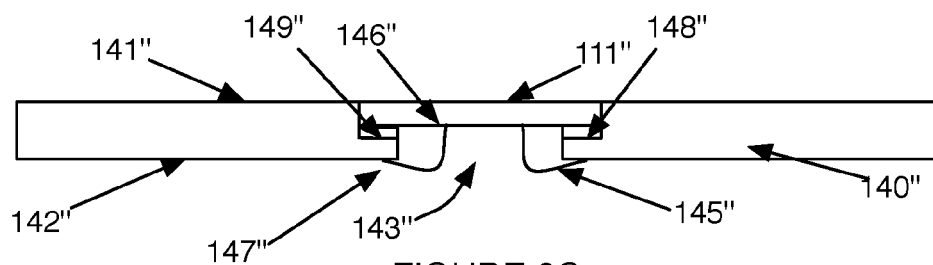


FIGURE 6C

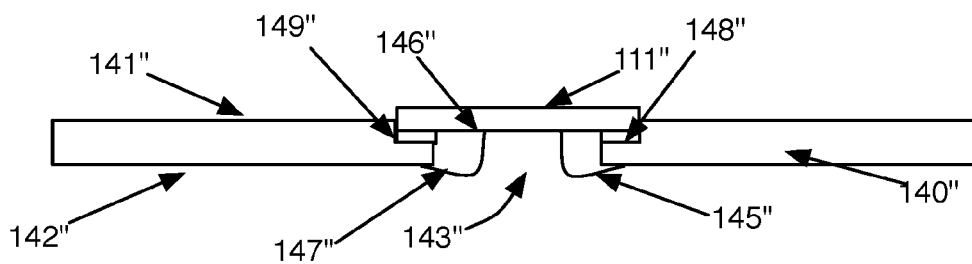


FIGURE 6D

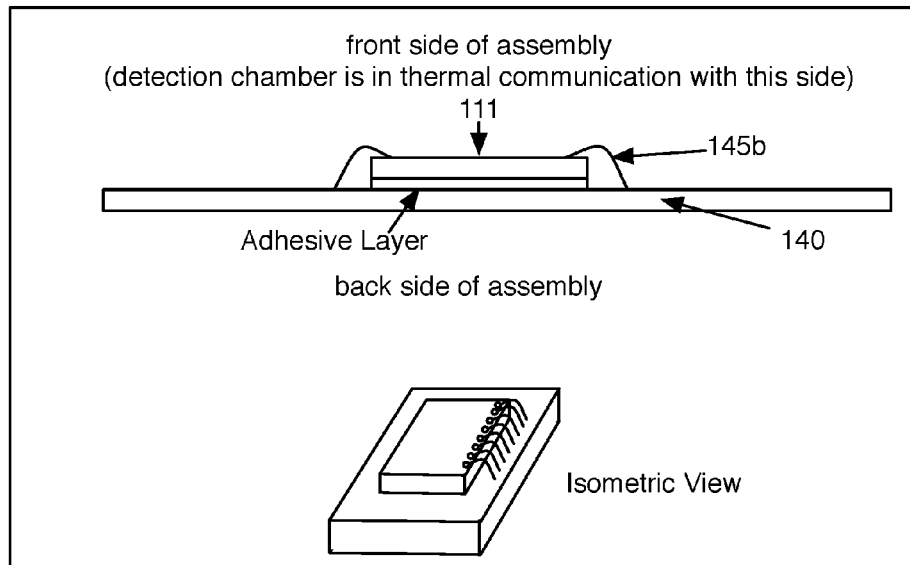


FIGURE 7A

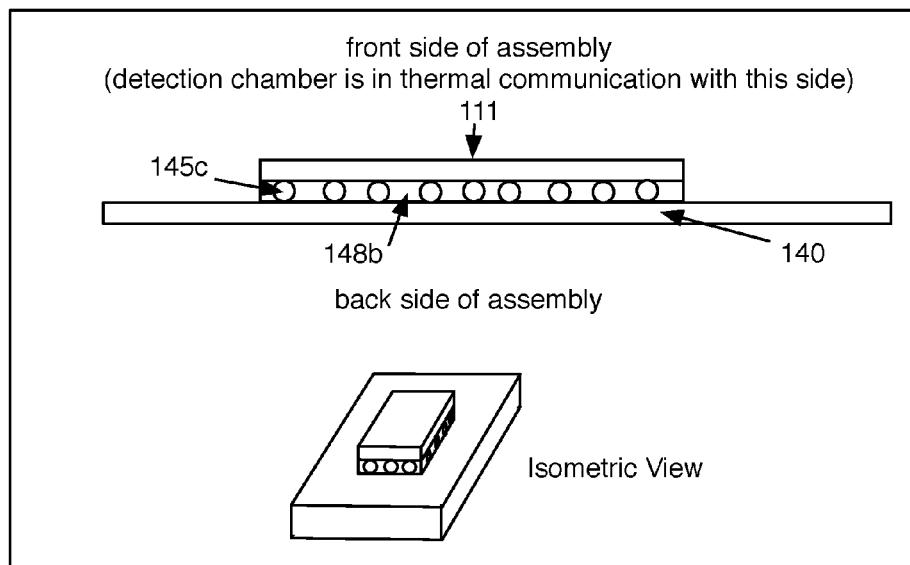
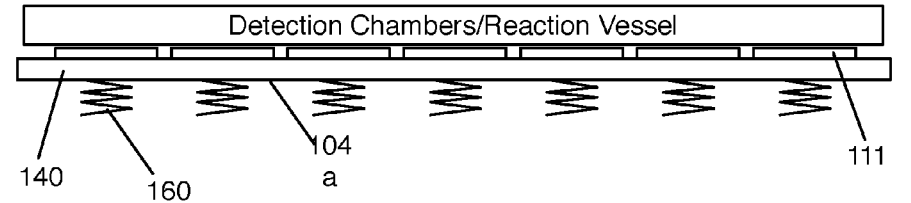
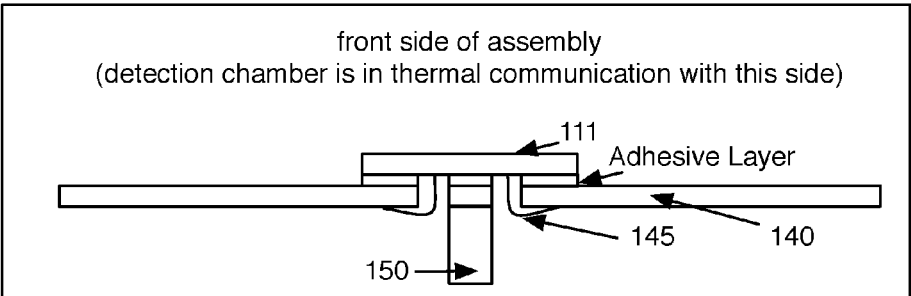
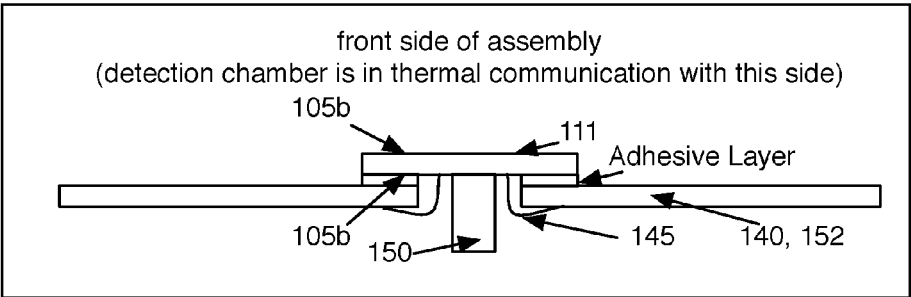


FIGURE 7B



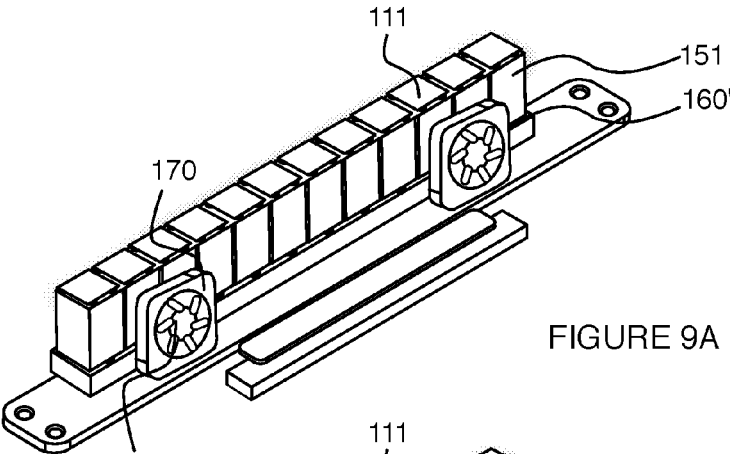


FIGURE 9A

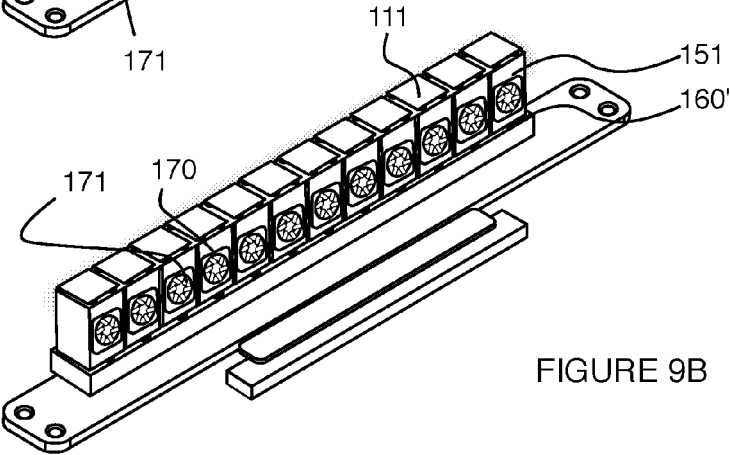


FIGURE 9B

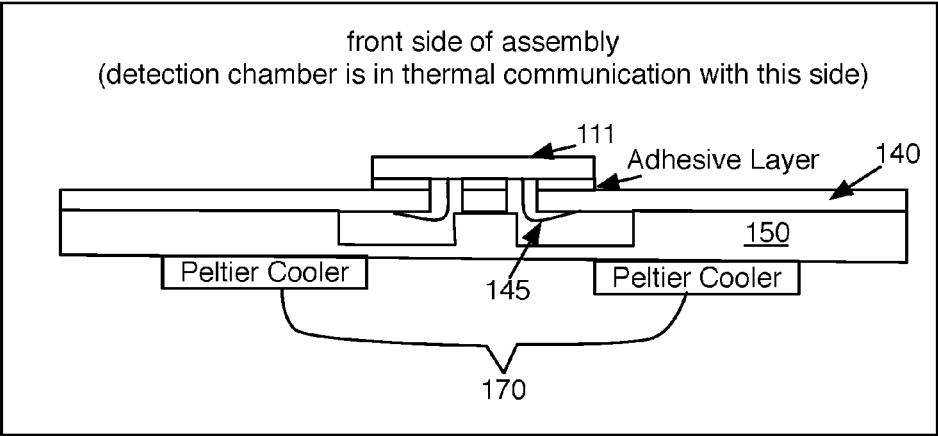


FIGURE 9C

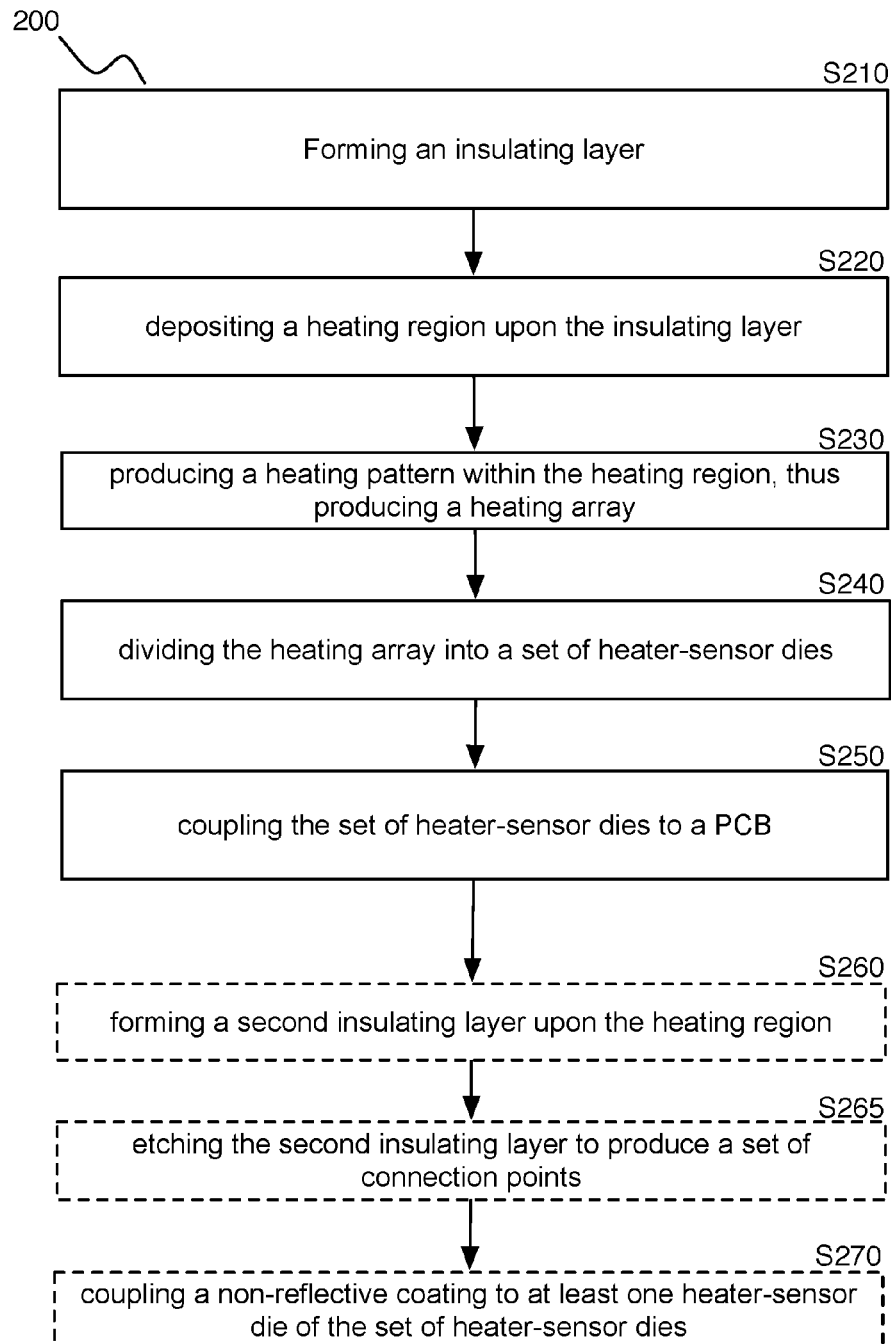


FIGURE 10

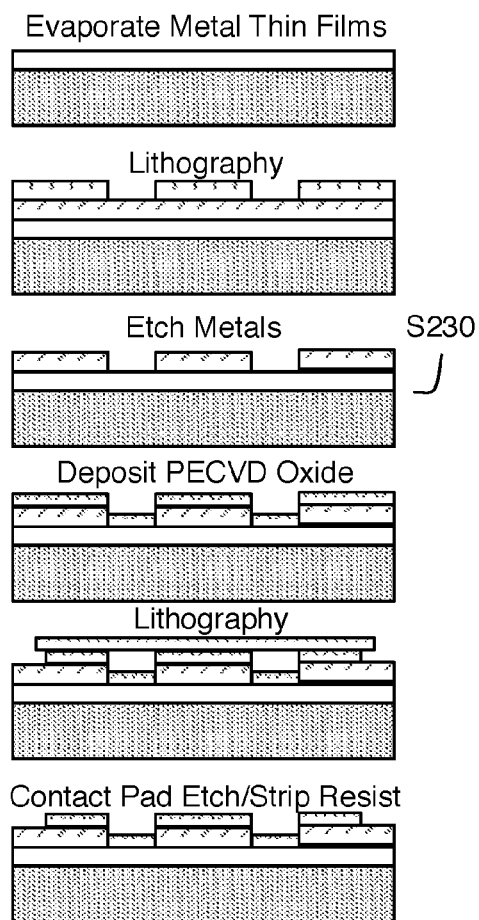


FIGURE 11A

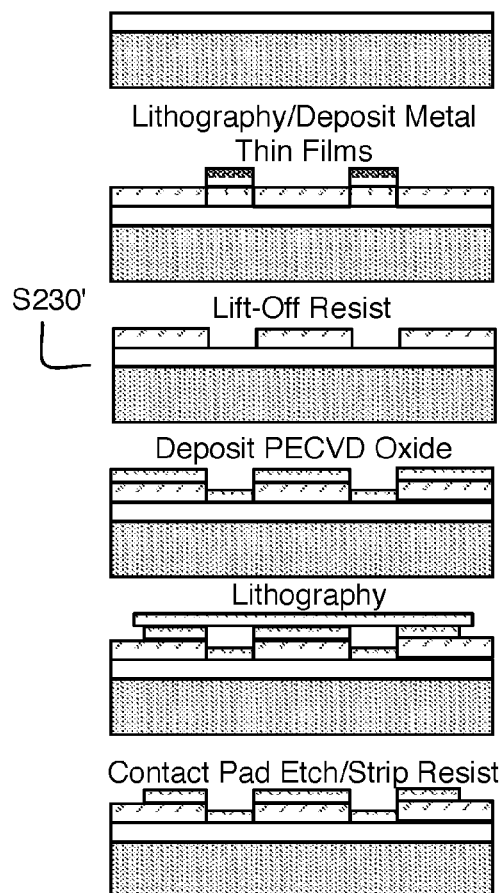


FIGURE 11B

US 10,226,771 B2

1

**THERMOCYCLING SYSTEM AND
MANUFACTURING METHOD****CROSS-REFERENCE TO RELATED
APPLICATIONS**

This application is a continuation of U.S. patent application Ser. No. 14/487,837, filed 16 Sep. 2014, which claims the benefit of U.S. Provisional Application Ser. No. 61/879,517 filed 18 Sep. 2013, both of which are incorporated in their entirety herein by this reference. This application is also related to U.S. application Ser. No. 14/487,808 filed 16 Sep. 2014, which is incorporated herein in its entirety by this reference.

TECHNICAL FIELD

This invention relates generally to the molecular diagnostics field, and more specifically to an improved sample thermocycling system and assembly method thereof.

BACKGROUND

Molecular diagnostics is a clinical laboratory discipline that has developed rapidly during the last 25 years. It originated from basic biochemistry and molecular biology research procedures, but now has become an independent discipline focused on routine analysis of nucleic acids (NA), including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for diagnostic use in healthcare and other fields involving analysis of nucleic acids. Molecular diagnostic analysis of biological samples can include the detection of one or more nucleic acid materials present in the specimen. The particular analysis performed may be qualitative and/or quantitative. Methods of analysis typically involve isolation, purification, and amplification of nucleic acid materials, and polymerase chain reaction (PCR) is a common technique used to amplify nucleic acids. Often, a nucleic acid sample to be analyzed is obtained in insufficient quantity, quality, and/or purity, hindering a robust implementation of a diagnostic technique. Current sample processing methods and molecular diagnostic techniques are often labor/time intensive, low throughput, and expensive, and systems of analysis are insufficient.

A rapid and efficient thermocycling system that can reliably thermocycle reagents used for processing of nucleic acids can significantly improve the efficiency and effective implementation of molecular diagnostic techniques, such as realtime polymerase chain reaction (RT-PCR). Microfabrication techniques can produce such thermocycling systems comprising precision heaters with low thermal masses and with well-coupled temperature sensors. However, challenges are inherent in ensuring that the microfabrication and assembly processes utilized to fabricate thermal cycling elements are extremely robust and reliable.

Due to these challenges and deficiencies of current molecular diagnostic systems and methods, there is thus a need for an improved sample thermocycling system and assembly method thereof. This invention provides such a system and assembly method.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B depict embodiments of a thermocycling system;

2

FIG. 2 depicts an example schematic of a heater-sensor die during fabrication in an example of a thermocycling system;

FIG. 3 depicts an example of heating and sensing elements in an example of a thermocycling system;

FIG. 4 depicts examples of additional elements of an embodiment of a thermocycling system;

FIGS. 5A and 5B depict examples of reverse wire bonding in embodiments of a thermocycling system;

FIGS. 6A-6D depict examples of reverse wire bonding in embodiments of a thermocycling system;

FIGS. 7A-7B depict variations of configurations of elements in an embodiment of a thermocycling system;

FIGS. 8A-8C depict variations of configurations of elements in an embodiment of a thermocycling system;

FIGS. 9A-9C depict variations of configurations of additional elements in an embodiment of a thermocycling system;

FIG. 10 depicts a flowchart of a method for assembling an embodiment of a thermocycling system; and

FIGS. 11A-11B depict variations of a method for assembling an embodiment of a thermocycling system.

**DESCRIPTION OF THE PREFERRED
EMBODIMENTS**

The following description of the preferred embodiments of the invention is not intended to limit the invention to these preferred embodiments, but rather to enable any person skilled in the art to make and use this invention.

1. System

As shown in FIGS. 1A and 1B, an embodiment of a sample thermocycling system **100** comprises: a set of heater-sensor dies **110**; an electronics substrate **140** configured to couple heating elements and sensing elements of the set of heater-sensor dies to a controller; a set of heat sink supports **150** coupled to at least one of the electronics substrate and the set of heater-sensor dies; and a set of elastic elements **160** coupled to the electronics substrate and configured to bias each of the set of heater-sensor dies against a detection chamber. In some embodiments, the system **100** further comprises a controller **165** coupled to the electronics substrate and configured to automate and/or control relevant heating parameters of the system **100**, and can further comprise a cooling subsystem **170** configured to dissipate heat from the system **100**. The system **100** functions to enable rapid thermocycling of samples while providing uniform heating and preventing mechanical failure of the system **100** during thermocycling. In specific applications, the system **100** can be used to rapidly and controllably thermocycle nucleic acid samples during performance of molecular diagnostic amplification techniques (e.g., PCR, RT-PCR), signal amplification techniques (e.g., bDNA, hybrid capture), and analytical techniques (e.g., gel electrophoresis, mass spectrometry). In some variations, the system **100** can be integrated into a molecular diagnostic system, such as that described in U.S. Pub. No. 2013/0210015, entitled "System and Method for Processing and Detecting Nucleic Acids", and filed on 13 Feb. 2013; however, the system **100** can additionally or alternatively be used with any other suitable system for processing biological or non-biological samples.

1.1 Heater-Sensor Dies

The set of heater-sensor dies **110** functions to controllably heat individual sample volumes. Preferably, each heater-sensor die **111** is a thin-film die that can be deposited onto another substrate (e.g., silicon substrate, glass substrate) that

can be packaged onto an electronics substrate **140**; however, each heater-sensor die **111** can alternatively comprise any suitable geometry and/or configuration that enables controlled, uniform, and rapid sample heating of a detection chamber in thermal communication with the heater-sensor die **111**. In some embodiments, the detection chambers can be those described in U.S. Pub. No. 2013/0209326, entitled “Microfluidic Cartridge for Processing and Detecting Nucleic Acids” and filed on 13 Feb. 2013, which is herein incorporated in its entirety by this reference; however, the detection chambers can alternatively be any other suitable container for processing a biological sample. Preferably, each heater-sensor die **111** is characterized by a small profile (e.g., <10 mm dimension), which ensures that the heater-sensor die **111** is able to thermocycle extremely rapidly; however, a heater-sensor dies **111** can alternatively be characterized by any suitable profile. Additionally, each heater-sensor die **111** is preferably configured to conform to a detection chamber (e.g., sample tube, sample container, sample heating zone of a cartridge for processing samples) configured to contain a sample during heating; however, a heater-sensor die **111** in the set of heater-sensor dies **110** can alternatively not conform to a sample container. In one variation, each heater-sensor die **111** can be coupled to a thermally conductive element (e.g., 600 micronx5x5 mm silicon spacer) using thermally conductive grease or another suitable material. In this variation, a connection between a heater-sensor die is thus protected against failure due to shear forces that can result from placement of a sample container on a heater-sensor die **111**. Other variations of preventing connection failure are described in Section 1.2 below.

Preferably, each heater-sensor die **111** in the set of heater sensor dies **110** comprises an insulating layer **112** that functions to provide an insulating barrier to isolate the heaters and sensors and a heating region **113** that functions to provide uniform sample heating, as shown in FIG. 2. The insulating layer **112** is preferably electrically insulating, but can additionally be thermally insulating. Furthermore, each heater-sensor die **111** preferably comprises two insulating layers **112** that are configured to “sandwich” the heating region **113**, thus isolating the heating region **113**; however, each heater-sensor die **111** can alternatively comprise any suitable number of insulating layers **112** arranged relative to the heating region **113** in any suitable manner. The heating region **113** preferably comprises a heating element **114** with an integrated sensing element **115**, and is composed of a metal or metal alloy. Furthermore, the heating region **113** is preferably defined by a pattern defined by geometric features (e.g., width, thickness, length, spacing) that facilitate uniform heating. However, in variations, the heating region **113** can alternatively not comprise an integrated sensing element **115**, can comprise any suitable number of heating elements **114**/sensing elements **115**, and/or can be composed of any other suitable material.

In a first specific example of a heater-sensor die **111**, as shown in FIG. 3, a heater-sensor die **111** is configured to uniformly heat a circular region having a diameter of 5 mm, spans a region of ~8.6 mmx7 mm, and comprises three heating elements **114a**, **114b**, **114c**: a central circular heating element **114a** and two circumferential heating elements **114b**, **114c** configured to form a boundary about the central circular heating element. The heater-sensor die **111** in the first specific example further comprises three integrated sensing elements **115a**, **115b**, **115c** (i.e., resistance temperature sensors, RTDs) distributed at three locations within the 5 mm circular region. In the first specific example, the

heating region was **113** etched away in a boustrophedonic pattern, designed using a layout editor (e.g., Mentor Graphics™ or L-Edit™), to form the heating surface. The heating elements **114** are defined by coarser patterning, and the sensing elements **115** are defined by finer patterning, as shown in FIG. 3A. Other variations and examples of the heater-sensor dies **111** can comprise any suitable patterning configuration and/or any suitable arrangement of insulating layer(s) **112a**, **112b** and heating region(s) **113**.

1.2 Other System Elements

As shown in FIGS. 1A and 1B, the system **100** further comprises an electronics substrate **140** and a set of heat-sink supports **150**. Furthermore, the system **100** can additionally comprise a controller **165** configured to automate and/or control relevant heating parameters of the system **100**, and/or a cooling subsystem **170** configured to dissipate heat from the system **100**, as shown in FIGS. 9A and 9B.

The electronics substrate **140** is preferably coupled to the set of heater-sensor dies **110**, and functions to enable communication between each heater-sensor die **111** in the set of heater sensor dies **110** and a controller **165**. The electronics substrate **140** preferably comprises a printed circuit board (PCB), and in some variations, comprises a flexible PCB, as shown in FIG. 4, in order to facilitate contact between heater-sensor dies **111** in the set of heater-sensor dies **110** and detection chambers (e.g., reaction vessels, detection chambers) for processing according to molecular diagnostic protocols. Alternatively, the electronics substrate **140** can alternatively comprise a rigid PCB or any other suitable PCB. Furthermore, the system **100** can comprise any suitable number of PCBs. Preferably, the set of heater-sensor dies **110** is assembled onto the electronics substrate in a manner that provides thermal and/or electrical isolation of each die **111** from the neighboring die(s), in particular, for variations wherein the electronics substrate **140** is characterized as having poor conductivity. However, the electronics substrate **140** and the set of heater-sensor dies **110** can be configured in any alternative suitable manner that provides isolation of each die **111**.

Preferably, the electronics substrate **140** is configured to couple to a heater-sensor die **111** by a reverse wire bond **145** coupled between a first connection point **146** (i.e., contact pad) of a set of connection points **46** on the heater-sensor die **111** and a second connection point **147** (i.e., pad) on the electronics substrate **140**, as shown in FIGS. 4 and 5A-5B. The reverse wire bond **145** functions to prevent unbonding of a heater-sensor die **111** from the electronics substrate **140** that can result from shear forces on at least one of the heater-sensor die **111** and the wire bond **145** and/or fatigue of the wire bond **145** during thermocycling. The reverse wire bond **145** can be made from a back-side of the electronics substrate **140**, in the orientation shown in FIGS. 5A-5B, through an aperture **143** defined within the electronics substrate **140**. The aperture **143** can be a single aperture, or a set of apertures corresponding to the set of heater-sensor dies **110**, and furthermore, multiple heater-sensor dies **111** of the set of heater-sensor dies **110** can be configured to couple to the electronics substrate **140** through an aperture **143** defined within the electronics substrate **140**. As such, the mappings between pads on the heater-sensor dies **110** and the electronics substrate **140** can be one-to-one or many-to-one in variations of coupling. In one variation, a set of apertures can be longitudinally spaced across the electronics substrate **140**; however, in other variations, the set of apertures can be distributed across the electronics substrate **140** in any other suitable manner. Also shown in FIGS. 5A-5B, coupling between the electronics substrate **140** and a heater-

US 10,226,771 B2

5

sensor die 111 can additionally comprise an adhesive layer 148 comprising cyanoacrylate and/or any other suitable adhesive material configured between the electronics substrate 140 and the heater-sensor die 111. In variations of heater-sensor die 111 coupling to the electronics substrate 140 with an adhesive layer 148, the adhesive layer is preferably heat resistance in order to prevent failure at the adhesive layer 148 during thermocycling.

In a first variation, as shown in FIG. 6A, a heater-sensor die 111 is configured to couple to a first side 141 of the electronics substrate 140 by a wire bond 145 that passes through an aperture 143 defined within the electronics substrate 140, such that the wire bond 145 couples at one end to a second side 142 of the electronics substrate 140. In this variation, a first connection point 146 on a surface of the heater-sensor die 111 closer to the first side 141 of the electronics substrate 140 is coupled to a second connection point 147 on the second side of the electronics substrate 140, by way of the aperture 143 and the reverse wire bond 145. In the first variation, the heater-sensor die 111 can be further stabilized in place by an adhesive layer 148 located between the first side 141 of the electronics substrate 140 and the heater-sensor die 111. Furthermore, while one wire bond 145 is described, the electronics substrate 140 can include a set of connection points distributed at regions of the second substrate surface between adjacent apertures of a set of apertures of the electronics substrate 140.

In a second variation, as shown in FIG. 6B, a heater-sensor die 111' is configured to couple to a first side 141' of the electronics substrate 140' by a wire bond 145' that passes into an aperture 143' defined within the electronics substrate 140. In this variation, the wire bond 145 couples, at a first connection point 146', to a surface of the heater-sensor die 111 closer to the first side 141' of the electronics substrate 140' and terminates at a second connection point 147' part-way between a first side 141' and a second side 142' of the electronics substrate 140', such that the wire bond 145' is not exposed at the second side of the electronics substrate 140'. In the second variation, the heater-sensor die 111' can also be stabilized in place by an adhesive layer 148' located between the first side 141' of the electronics substrate 140' and the heater-sensor die.

In a third variation, as shown in FIGS. 6C and 6D, a heater-sensor die 111" is configured to rest within a recess 149" at the first side of the electronics substrate 140", wherein the recess 149" is connected to (e.g., contiguous with) an aperture 143" defined within the electronics substrate 140". As shown in FIG. 6C, the recess 149" can be configured such that a heating surface of the heater-sensor die 111 is flush with a first surface 141" of the electronics substrate 140"; however, in an example shown in FIG. 6D, the recess can also be configured such that a heating surface of the heater-sensor die 111" is not flush with the first surface 141" of the electronics substrate 140". In the third variation, the wire bond 145" couples, at a first connection point 146", to a surface of the heater-sensor die 111" partially situated within the electronics substrate 140", and terminates at a second connection point 147" either part-way between the first side 141" and a second side 142" of the electronics substrate 140" (as in the second variation), or at a second point 147" at the second side 142" of the electronics substrate 140" (as in the first variation). Thus, in the third variation, a surface of the heater-sensor die 111" is stabilized within the recess 149" of the electronics substrate 140" to further prevent shearing or other forms of mechanical failure that could compromise coupling between the heater-sensor die 111" and the electronics substrate 140". In the third

6

variation, the heater-sensor die 111" can also be further stabilized within the recess 149" by an adhesive layer 148" between the recess 149" and the heater-sensor die 111". While a single recess is described, the electronics substrate 140" can include a set of recess, each contiguous with at least one aperture of set of apertures of the electronics substrate 140.

Other variations of the reverse wire bond(s) 145 between a heater-sensor die 111 and the electronics substrate 140 can comprise any suitable combination of the above variations, and can additionally or alternatively comprise any suitable encapsulation, embedding, or potting of wire bonds to further prevent failure in the wire bonds.

In still other variations, each heater-sensor die 111 can be coupled to the electronics substrate 140 by any other suitable method. In one variation, the coupling can comprise a "top-side" wire bond 145b, as shown in FIG. 7A. In this variation, a thin wire (e.g., 10-300 microns thick) composed of an electrically conductive material (e.g., aluminum, gold, or copper wire) is coupled between a first connection point 146 on the heater-sensor die 111 and a second connection point 147 on a second side 142 of the electronics substrate 140, in the orientation shown in FIG. 7A. Furthermore, in this variation, the "top-side" wire bonds 145b are potted, embedded, and/or encapsulated to protect them from mechanical failure. In another variation, the coupling can comprise a flip-chip bond 145c, as shown in FIG. 7B. In this variation, a volume (e.g., ball) of solder is placed between a first connection point 146 on a heater-sensor die 111 and a second connection point 147 on the electronics substrate 140. Furthermore, in this variation, a filler material 148b can be placed in regions between the electronics substrate 140 and the heater-sensor die 111 not connected by a volume of solder of the flip-chip bond 145c. In other variations, the coupling can additionally or alternatively comprise any suitable adhesive (e.g., cyanoacrylate adhesive) layer 148 configured between the heater-sensor die 111 and the electronics substrate 140.

The set of heat sink supports 150 is preferably coupled to at least one of the set of heater-sensor dies 110 and the electronics substrate 140 and functions to facilitate rapid thermocycling by dissipating heat from the set of heater-sensor dies 110 and/or the electronics substrate 140. The set of heat sink supports 150 can further function to provide structural support for the set of heater-sensor dies 110, such that the set of heater-sensor dies 110 is supported during compression (e.g., compression against a set of detection chambers) and/or tension. In the absence of heat sinking, the electronics substrate 140 and the surrounding environment can potentially retain too much heat, which compromises the cooling of the set of heater-sensor dies 110. The set of heat sink supports 150 can comprise multiple heat sink supports 151 configured to define any suitable number of contact locations, or can alternatively comprise a single heat sink support 151 configured to define any suitable number of contact locations. As shown in FIGS. 6A and 6B, the system 100 preferably couples to a detection chamber (e.g., reaction vessel, detection chamber) from a first side 101a of the system, which can restrict heat dissipation from the first side 101a of the system. Furthermore, the second side 101b of the system 100 is typically used for optical imaging for monitoring (e.g., realtime monitoring, delayed monitoring), and further limiting heat-sinking from the second side 101b. Thus, it is preferable for the set of heat sink supports 150 to couple to the system 100 from a side of the system 100 that does not physically interfere with optical imaging apparatus interfacing with the system 100. However, alternative con-

figurations of the set of heat sink supports **150** can comprise coupling at any suitable side and/or any number of sides of the system **100**.

As shown in FIGS. **8A** and **8B**, the set of heat sink supports **150** can be configured in any of a number of variations. In a first variation, each heat sink support **151** can be directly placed against a first surface **105a** of heater-sensor die **111** opposing that of a second surface **105b** contacting a detection chamber, as shown in FIG. **7A**. The first variation enables efficient transfer of heat out of the first surface **105a** of the heater-sensor die away from a respective detection chamber; however, excessive heat sinking can affect heating ramp rates. In a second variation, the system **100** comprises a thermally insulating assembly **152** between a heater-sensor die **111** and a corresponding heat sink support **151**, as shown in FIG. **7B**. In the second variation, the electronics substrate **140** can serve as the thermally insulating assembly **152** and can be situated between the heater-sensor die **111** and a heat sink support **151**. Furthermore, in the second variation, a suitable thermal resistance provided by the electronics substrate **140** (e.g., through thickness, material selection, a combination of features) could produce a thermal couple between the heater-sensor die **111** and the heat sink support **151** to permit the heating capacity of the heater-sensor die **111** to achieve the heating times and/or heating ramp rate required by the application, while still allowing adequate cooling rates. Additionally, the second variation can provide increased backside support to each of the set of heater-sensor dies **110** as well as increased surface for adhesion.

In specific examples of the second variation, heat sinking and supporting the “backside” of the electronics substrate **140** can be implemented across multiple heater-sensor dies **111**, separated by Society for Laboratory Automation and Screening (SLAS) standard spacings, such as 9 mm, 4.5 mm or 2.25 mm spacings. The heat sink support **151** material (e.g., aluminum, copper, silver) in the specific examples is mated with the electronics substrate **140** at each heater-sensor die location, with an air gap positioned laterally between each heater-sensor die location. This configuration can further function to reduce cross talk across a set of detection chambers in contact with the set of heater-sensor dies **110**. The set of heat sink supports **150** can, however, be configured in any other suitable manner to provide heat dissipation within the system **100**, without obstruction of optical detection apparatus, and with provision of desired heat ramping and/or cycling behavior.

In specific examples of the second variation, heat sinking and supporting the backside (i.e., first side **141**) of the electronics substrate **140** can be implemented across multiple heater-sensor dies **111**, separated by Society for Laboratory Automation and Screening (SLAS) standard spacings, such as 9 mm, 4.5 mm or 2.25 mm spacings. The heat sink support **151** material (e.g., aluminum, copper, silver) in the specific examples is mated with the electronics substrate **140** at each heater-sensor die location, with an air gap between locations. This configuration can further function to reduce cross talk between at least a first detection chamber and a second detection chamber interfacing with the system **100**.

The set of elastic elements **160** is preferably coupled to a first surface **104a** of the electronics substrate **140**, and functions to promote contact between the set of heater-sensor dies **110** and detection chambers (e.g., reaction vessels, detection chambers) for sample processing according to molecular diagnostic protocols. The set of elastic elements **160** can comprise any one more of springs and elastomeric elements, which can deform and provide transmit a biasing

force, through the electronics substrate **140**, to reinforce contact between a set of detection chambers and the set of heater-sensor dies **110**. The set of elastic elements **160** can, however, additionally or alternatively include any other suitable elements configured to provide a biasing force that reinforces contact between a set of detection chambers and the set of heater-sensor dies **110** in an elastic or a non-elastic manner. In one such alternative variation, the system **100** can include one or more actuators configured to drive each of the set of heater-sensor dies **111** toward a corresponding detection chamber, and in another such alternative variation, the system **100** can include a set of magnets (e.g., including magnet pairs surrounding the set of heater-sensor dies **110** and a corresponding set of detection chambers), that function to reinforce coupling between the set of heater-sensor dies **110** and the set of detection chambers. However, any other suitable elements can additionally or alternatively be used to facilitate uniform and consistent coupling between the set of heater-sensor dies **110** and a set of detection chambers.

In embodiments of the system **100** including a set of elastic elements **160**, the set of elastic elements **160** is preferably coupled to a first surface **104a** of the electronics substrate **140**, as shown in FIG. **8C**, such that each elastic element in the set of elastic elements **160** facilitates contact between a heater-sensor die **111** and a corresponding detection chamber. In a first variation, the set of elastic elements **160** is coupled to first surface **104a** of a flexible PCB of the electronics substrate **140**, as shown in FIG. **5A**. In the first variation, contact between each heater-sensor die **111** and a corresponding detection chamber is thus maintained by a biasing force provided by an individual spring through the flexible PCB of the electronics substrate **140**. In the first variation, the number of elastic elements in the set of elastic elements **160** is equal to the number of heater-sensor dies in the set of heater-sensor dies **110**, such that the set of elastic elements **160** and the set of heater-sensor dies **110** are paired in a one-to-one manner. Alternatives to the first variation can, however, comprise any suitable number of elastic elements in relation to a number of heater-sensor dies **110**. In a second variation, the set of heater-sensor dies **110** is coupled to a second surface **104b** of a rigid PCB of the electronics substrate **140**, with the set of elastic elements **160** coupled to the first surface **104a** of the rigid PCB. In the second variation, the set of elastic elements **160** thus functions to collectively transfer a force through the rigid PCB to maintain contact between the set of heater-sensor dies **110** and the detection chambers. Alternatives to the second variation can also comprise any suitable number of springs in relation to a number of heater-sensor dies in the set of heater-sensor dies **110**. Furthermore, variations of the system **100** can include one or more elastic elements coupled to any other elements directly or indirectly coupled to the set of heater-sensor dies **110**. For instance, the system **100** can additionally or alternatively include one or more springs **160'** coupled to base surfaces of the set of heat-sink supports **150** interfacing with the set of heater-sensor dies, in order to transmit biasing forces.

As shown in FIG. **1**, the system **100** can further comprise a controller **165**, which functions to automate and/or control heating parameters provided by the set of heater-sensor dies **110**. The controller **165** can further be configured to provide heat parameter output commands to the heating element(s) **114**, and/or configured to receive communication of heating parameters (e.g., detected temperatures) sensed at the sensing element(s) **115** of the system **100**. The controller **165** preferably comprises a proportion-integral-derivative (PID)

controller, but can alternatively be any other suitable controller **165**. The controller **165** preferably interfaces with the set of heater-sensor dies **110** through the electronics substrate **140** by a connector; however, the controller **165** can interface with the set of heater-sensor dies **110** in any alternative suitable manner. Preferably, the controller **165** is configured to automate and control heat output parameters, including any one or more of: heating temperatures, heating ramp rates, heating times (e.g., holding times), and any other suitable heating parameter(s). Furthermore, the controller **165** can be configured to control individual heater-sensor dies **111** in order to provide unique heating parameters for individual detection chambers and/or can be configured to provide common heating parameters for all heater-sensor dies **111** in the set of heater-sensor dies **110**. In a specific example, the controller **165** comprises a Yokogawa UT750 PID controller, an Arduino UNO R3 microcontroller configured to cycle the UT750 through temperature stages and to control temperature holding, a resistance-to-voltage conversion circuit, and two power supplies—a first power supply configured to supply power to the set of heater-sensor dies **110** and a second power supply configured to supply voltage to the resistance-to-voltage conversion circuit. In the specific example, the controller **165** comprises a resistance-to-voltage conversion circuit because the UT750 PID controller requires voltage as an input for PID control. In another specific example, the controller **165** comprises a National Instruments LabView based system comprised of an NI cDAQ-9178 chassis with an NI 9219 universal analog input card and an NI 9485 eight-channel solid-state relay sourcing or sinking digital output module solid-state relay card. In this specific example, the cDAQ-9178 supports the NI 9219 and NI 9485 cards, the NI 9219 is used to obtain the RTD inputs, and the NI 9485 cycles the power supply voltage to individual heater-sensor dies of the set of heater-sensor dies **110**. Further, in this specific example, the controller **165** is expandable to 12 or more channels through the use of additional NI 9219 and NI 9485 cards, each of which can handle several channels.

As shown in FIGS. 9A and 9B, the system **100** can further comprise a cooling subsystem **170**, which functions to provide heat transfer from the system **100** in order to further enhance controlled heating and cooling by the system **100**. The cooling subsystem **170** is preferably configured to provide at least one of convective cooling and conductive cooling of the system **100**, but can alternatively be configured to provide any other suitable cooling mechanism or combination of cooling mechanisms. In one variation, the cooling subsystem **170** can comprise a fan **171** that provides convective heat transfer from the system **100**. In this variation, the fan **171** can be coupled to any suitable element of the system **100**, such as the set of heat sink supports **150**, as shown in FIG. 9A. Furthermore, alternatives to this variation can comprise any suitable number of fans of any suitable dimension and configuration, examples of which are shown in FIGS. 9A and 9B. In one such example, the system can include a set of cooling elements integrated with each heat sink support of the set of heat sink supports. In another variation, the cooling subsystem **170** can additionally or alternatively comprise a Peltier device, as shown in FIG. 9C. The Peltier device can be cooled and maintained at a defined temperature (e.g., in the 10-25 C range) to provide a substantial temperature gradient for cooling during a thermal cycling process, which can decrease cooling times and/or cycle times. In yet another variation, the cooling subsystem **170** can additionally or alternatively comprise a liquid cooling system (e.g., water cooling system) configured to

surround and absorb heat from one or more heater-sensor dies of the set of heater-sensor dies **110**, for instance, by way of the set of heat sink supports **150**. The cooling subsystem **170** can additionally or alternatively comprise any other suitable cooling element(s).

In some variations, reflection from the set of heater-sensor dies **110** can interfere with light transmitted to photodetectors of an optical subsystem opposed (e.g., directly opposed, in opposition) to the set of heater-sensor dies **110** (e.g., light emitted from the set of biological samples, light transmitted through filters of an optical subsystem), especially in configurations wherein a set of detection chambers is configured between the set of heater-sensor dies and optical elements of an optical subsystem. In these variations, the set of heater-sensor dies **110** can include elements that reduce or eliminate reflection from any portion of the set of heater-sensor dies (e.g., reflection from the heating region, etc.), thereby facilitating analysis of a set of biological samples within the set of detection chambers. In one variation, the set of heater-sensor dies **110** can include or be coupled to one or more non-reflective coatings **180** at surfaces of the set of heater-sensor dies **110** upon which light from the optical subsystem impinges. In a specific example, the non-reflective coating **180** can comprise a high-temperature paint (e.g., dark paint, flat paint) that functions to absorb and/or diffuse light from an opposing optical subsystem, while facilitating heat transfer to a set of detection chambers in thermal communication with the set of heater-sensor dies **110**. In another variation, the set of heater-sensor dies **110** can be configured to be in misalignment with photodetectors of the optical subsystem, such that reflection does not interfere with light transmitted to the photodetectors of the optical subsystem. In one example, the set of heater-sensor dies **110** can be configured to heat a set of detection chambers from a first direction, and the optical subsystem can be configured to receive light from the set of detection chambers from a second direction (e.g., a direction non-parallel to the first direction), such that reflection from the set of heater-sensor dies **110** does not cause interference. In still other variations, the set of heater-sensor dies **110** can include any other suitable elements (e.g., coatings, layers, etc.) and/or be configured in any other suitable manner that eliminates, prevents, or mitigates reflection from the set of heater-sensor dies **110** from interfering with light transmitted to photodetectors of an optical subsystem in opposition to the set of heater-sensor dies **110**.

Variations of the system **100** can, however, include any other suitable element(s) configured to provide uniform, accurate, precise, and reliable heating of one or more detection chambers in thermal communication with the system **100**. Furthermore, as a person skilled in the art will recognize from the previous detailed description and from the figures, modifications and changes can be made to the preferred embodiments of the system **100** without departing from the scope of the system **100**.

2. Method of Assembly

As shown in FIG. 10, a method **200** of assembling an embodiment of a thermocycling system **100** comprises forming a first insulating layer coupled to exposed surfaces of a substrate **S210**; depositing a heating region upon the first insulating layer **S220**; producing a heating pattern within the heating region, thus producing a heating array **S230**; dividing the heating array into a set of heater-sensor dies **S240**; and coupling the set of heater-sensor dies to a electronics substrate **S250**. The method **200** can further comprise forming a second insulating layer upon the heating region **S260**, which functions to electrically isolate the

US 10,226,771 B2

11

heating region on a first side and a second side. The method 200 functions to produce a thermocycling system 100, embodiments, variations, and examples of which are described above, wherein the thermocycling system 100 provides rapid and uniform thermocycling of samples and comprises elements configured to prevent mechanical failure.

Block S210 recites: forming a first insulating layer coupled to exposed surfaces of a substrate, and functions to generate a layer that electrically insulates the heating region deposited in Step S220. The substrate is preferably a silicon substrate, but can alternatively be any other suitable semi-conducting, or non-conducting substrate. As such, in variations, the substrate can be composed of a semi-conducting material (e.g., silicon, quartz, gallium arsenide), and/or an insulating material (e.g., glass, ceramic). In some variations, the substrate 130 can even comprise a combination of materials (e.g., as in a composite, as in an alloy). In examples wherein the substrate is a silicon substrate, the substrate can be composed of silicon with any suitable type (e.g., P-type), doping (e.g., boron-doping), miller index orientation, resistivity, thickness, total thickness variation, and/or polish.

In forming the first insulating layer, Block S210 can be performed using any one or more of: thermal oxide growth, chemical vapor deposition (CVD), spin coating, spray coating, and any other suitable method of depositing a localized layer of an insulating material. Preferably, the first insulating layer is composed of an insulating oxide material, and in examples can include any one or more of: a thermally grown silicon oxide, a chemical vapor deposited oxide, a deposited titanium oxide, a deposited tantalum oxide, and any other suitable oxide grown and/or deposited in any other suitable manner. However, the first insulating layer can additionally or alternatively include an insulating polymer (e.g., a polyimide, a cyanate ester, a bismaleimide, a benzoxazine, a phthalonitrile, a phenolic, etc.) that is chemical and heat resistant and/or any other suitable material (e.g., chemical vapor deposited nitride, other nitride, paralene, etc.) that is configured to provide the first insulating layer.

In one example of Block S210, the first insulating layer comprises an oxide material, and is formed by growing the oxide material on a substrate. In one example of Block S210, the insulating layer comprises a 0.2 mm layer of silicon oxide, and is formed on a 100 mm silicon wafer using thermal oxidation at 900° C. using water vapor (i.e., in wet oxidation) or oxygen (i.e., in dry oxidation) as the oxidant. In alternative variations and examples of Block S210, the first insulating layer can be formed using high or low temperature thermal oxidation, using any suitable oxidant, and/or using any other suitable method (e.g., fluid deposition of an electrically insulating polymer, softbaking/hardbaking of a deposited polymer, etc.).

Block S220 recites depositing a heating region upon the insulating layer, and functions to form a thermally conductive substrate that is robust during rapid thermocycling. Preferably, the heating region comprises a metal or a metal alloy and can comprise multiple layers; however, the heating region can alternatively comprise any suitable thermally conducting material, and can comprise any suitable number of layers. Additionally, the heating region is preferably deposited in a uniform layer; however, the heating region can be deposited non-uniformly in other variations. In one variation, the heating region comprises an adhesion material layer and a noble material layer, wherein the noble material layer is deposited upon the adhesion material layer after the adhesion material layer is deposited upon the first insulating

12

layer. In examples of this variation, the adhesion layer can comprise chromium or titanium, and the noble layer can comprise gold or platinum. In one example of Block S220, the conductive material(s) of the heating region is(are) deposited using an evaporation process; however, in other examples, the conductive material(s) can be deposited by sputtering, plating (e.g., chemical plating, electrochemical plating), or any other suitable method (e.g., electrodeposition). Furthermore, in examples wherein a heating region material is evaporated or sputtered, the insulating layer-substrate subassembly generated in Block S210 can be translated or rotated in order to facilitate uniform deposition of the heating region material.

Block S230 recites producing a heating pattern within the heating region, and functions to produce a heating array characterized by a heating pattern that provides uniform heating and desirable resistances for heating elements and/or sensing elements (e.g., RTDs) defined within the heating region. As such, Block S240 preferably produces a heating pattern having geometric features (e.g., width, thickness, length, spacing) that facilitate uniform heating and provide desired heating and sensing characteristics (e.g., resistance characteristics). In some variations, the pattern can define any one or more of: linear segments, non-linear segments, boustrophedonic segments, continuous segments, non-continuous segments, and any other suitable segment(s) having any other suitable geometry (i.e., width, depth, height, length, path, etc.) In a specific example, the heating pattern was designed using a layout editor (e.g., Mentor Graphics™ or L-Edit™), and comprises a boustrophedonic pattern that is coarse for heating elements and fine for sensing elements. In alternative variations, the heating pattern can be designed using any other suitable method, and can alternatively or additionally comprise any features that contribute to uniform heating and/or suitable resistance ranges. During implementation of Block S230, the heating pattern can be produced photolithographically using a positive resist process, as shown in FIG. 11A. In one example, the heating region can be covered with positive photoresist (e.g., a photomask designed according to the heating pattern) and lithographically etched in exposed regions. In the example, the positive photoresist can then be removed to reveal the heating pattern. In other variations, the heating pattern can be produced using any lithographic method, using positive and/or negative etching to form the heating pattern, and/or using any other suitable method. In one example of an alternative implementation of Step S230', the heating pattern can be produced using a lift-off process, as shown in FIG. 11B, wherein a sacrificial layer is used to define the heating pattern, the heating region material(s) is(are) deposited, and then the sacrificial layer is removed to reveal the heating pattern.

Block S240 recites dividing the heating array into a set of heater-sensor dies, and functions to divide the heating array into a set of heater-sensor dies configured to heat multiple detection chambers (e.g., reaction vessels, sample containers, wells of a plate, chambers of a cartridge) in parallel (e.g., simultaneously, in sequence). Block S240 can also comprise cleaning and drying the heating array prior to and/or after dividing the heating array into a set of heater-sensor dies. Upon completion of Block S240, individual heater-sensor dies of the set of heater-sensor dies can be coupled to one or multiple electronics substrates in order to provide uniform heating of individual sample containers with independent control of heating parameters provided at each of the set of heater-sensor dies. In Block S240, the heating array is preferably divided using a dicing method (e.g., mechanical

US 10,226,771 B2

13

dicing by saw, laser dicing, water cutting, stealth dicing, etc.), but can additionally or alternatively be divided using any other suitable method (e.g., dice before grind). Furthermore, the heating array is preferably divided into rectangular dies, but can alternatively be divided into dies of any suitable morphology (e.g., polygonal dies, non-polygonal dies, circular dies, ellipsoidal dies, etc.). In a specific example, as shown in FIG. 3A, each heater-sensor die produced after division of the heating array has dimensions of approximately 8.6 mm×7 mm, with a circular heating surface that is 5 mm in diameter.

Block S250 recites coupling the set of heater-sensor dies to an electronics substrate, and functions to provide a set of robust connections between the set of heater-sensor dies and an electronics substrate. Coupling in Step S250 comprises forming an electrical connection between connection points on the heater-sensor dies and the electronics substrate, which enables driving of a heating current from the electronics substrate to each of the set of heater-sensor dies (e.g., simultaneously, non-simultaneously). The electrical connection can be provided by a conducting wire (e.g., aluminum wire, gold wire, copper wire) of any suitable thickness (e.g., 10-200 microns), or by soldering. Furthermore, coupling in Block S250 preferably comprises coupling heater-sensor dies with a suitable center-to-center spacing that accommodates the spacing of detection chambers intended to be heated by the system. In a specific example, coupling in Block S250 comprises providing a center-to-center spacing between heater-sensor dies of 9 mm, 4.5 mm, or 2.25 mm according to Society of Laboratory Automation Standards (e.g., SLAS Microplate Standards).

Preferably, for a heater-sensor die, Block S250 comprises forming a reverse wire bond between a connection point (i.e., pad) on the heater-sensor die and a connection point (i.e., pad) on the electronics substrate, as shown in FIGS. 5A-5B and 6A-6D. The reverse wire bond prevents unbonding of a heater-sensor die from the electronics substrate, which can be caused due to mechanical forces on the wire bond and/or heater-sensor die, or fatigue failure of a connection. In Block S250, the reverse wire bond is preferably made from a back-side of the electronics substrate, in the orientation shown in FIGS. 5A-5B, through an aperture defined within the electronics substrate. The aperture can be a single aperture, or a set of apertures, and furthermore, multiple heater-sensor dies of the set of heater-sensor dies 110 can be configured to couple to the electronics substrate through an aperture defined within the electronics substrate. The mappings between pads on the heater-sensor dies and the electronics substrate can be one-to-one or many-to-one in variations of coupling. Also shown in FIGS. 5A-5B, Block S250 can additionally comprise depositing an adhesive layer comprising cyanoacrylate and/or any other suitable adhesive material between a heater-sensor die and the electronics substrate.

In a first variation, as shown in FIG. 6A, Block S250 comprises coupling a heater-sensor die to a first side of the electronics substrate by a wire bond that passes through an aperture defined within the electronics substrate, such that the wire bond couples at one end to a second side of the electronics substrate. In this variation, a connection point on a surface of the heater-sensor die closer to the first side of the electronics substrate is coupled to a connection point on the second side of the electronics substrate, by way of the aperture and the reverse wire bond. In the first variation of Block S250, the heater-sensor die can be further stabilized in place by depositing an adhesive layer at the first side of the electronics substrate.

14

In a second variation, as shown in FIG. 6B, Block S250 comprises coupling a heater-sensor die to a first side of the electronics substrate by a wire bond that passes into an aperture defined within the electronics substrate. In this variation, the wire bond is configured to couple, at one end, to a surface of the heater-sensor die closer to the first side of the electronics substrate, and configured to terminate at a connection point part-way between a first side and a second side of the electronics substrate, such that the wire bond is not exposed at the second side of the electronics substrate. In the second variation of Block S250, the heater-sensor die 111 can also be stabilized in place by depositing an adhesive layer at the first side of the electronics substrate.

In a third variation, as shown in FIGS. 6C-6D, Block S250 comprises providing a recess within the electronics substrate at a first side of the electronics substrate, and coupling (e.g., mounting) a heater-sensor die within the recess of the electronics substrate, wherein the recess is connected to (e.g., contiguous with) an aperture defined within the electronics substrate. Providing the recess of the third variation preferably comprises forming the electronics substrate with a recess and aperture contiguous with the recess, wherein examples of forming can include any one or more of: molding (e.g., injection molding), casting, printing (e.g., 3D printing), and any other suitable method of forming the electronics substrate. Providing the recess of the third variation can additionally or alternatively comprise a method of removing material from a substrate, such as etching, machining (e.g., drilling, milling), and any other suitable method of material removal.

As shown in FIG. 6C, a first example of providing the recess of Block S250 can include providing a recess that is configured such that a heating surface of the heater-sensor die is flush with a first surface of the electronics substrate; however, in an example shown in FIG. 6D, providing the recess can include providing a recess that is configured such that a heating surface of the heater-sensor die is not flush with the first surface of the electronics substrate. In the third variation of Block S250, the wire bond is configured to couple, at one end, to a surface of the heater-sensor die partially situated within the electronics substrate, and configured to terminate at a connection point either part-way between the first side and a second side of the electronics substrate (as in the second variation of Block S250), or at a termination point at the second side of the electronics substrate (as in the first variation of Block S250). Thus, the third variation of Block S250 comprises stabilizing a surface of the heater-sensor die within the recess of the electronics substrate to further prevent shearing or other forms of mechanical failure that could compromise a connection between the heater-sensor die and the electronics substrate. In the third variation, the heater-sensor die can also be further stabilized by providing an adhesive layer within the recess.

In other variations, Block S250 can comprise coupling each heater-sensor die to the electronics substrate by any other suitable method. In one such variation of Block S250, coupling can comprise a “top-side” wire bond, in the orientation shown in FIG. 7A. In this variation, Block S250 comprises coupling a thin wire (e.g., 10-300 microns thick) composed of an electrically conductive material (e.g., aluminum, gold, or copper wire) between a connection point on the heater-sensor die and a connection point on the top side electronics substrate (in the orientation shown in FIG. 7A). Furthermore, in this variation, the “top-side” wire bonds are potted, embedded, or encapsulated to protect them from mechanical failure. In another alternative variation, coupling

in Block S250 can comprise forming a flip-chip bond, as shown in FIG. 7B. In this variation, Block S250 can include providing a volume (e.g., ball) of solder configured between a connection point on a heater-sensor die and a connection point on the electronics substrate. Furthermore, in this variation, Block S250 can additionally comprise depositing a filler material within regions between the electronics substrate and the heater-sensor die not connected by a volume of solder, in order to further stabilize the assembly. In other variations, the coupling can additionally or alternatively comprise any suitable adhesive (e.g., cyanoacrylate adhesive).

Wire bonding in variations of Block S250 can comprise any suitable combination of the above variations, and can additionally or alternatively comprise any suitable encapsulation, embedding, or potting of wire bonds to further prevent failure in the wire bonds. Furthermore, while variations of Block S250 are described for coupling of a set of heater-sensor dies to an electronics substrate, Block S250 can alternatively comprise coupling of a single heater-sensor die to the electronics substrate, in order to produce a single heating surface configured to heat a detection chamber in thermal communication with the heater-sensor die. However, Block S250 can alternatively comprise coupling any suitable number of heater-sensor dies to any suitable number of electronics substrates.

As shown in FIG. 10, the method 200 can further comprise Block S260, which recites forming a second insulating layer upon the heating region. Block S260 functions to electrically isolate the heating region on a first side and a second side of the heating region, and is preferably performed prior to coupling of the set of heater-sensor dies to the electronics substrate. However, Block S260 can alternatively be performed at any other suitable time relative to other blocks of the method 200. Preferably, Block S260 comprises depositing (e.g., electrodepositing, using CVD) or growing (e.g., by thermal oxidation) an oxide on the heating region, such that the heating region is “sandwiched” between two oxide layers; however, Block S260 can additionally or alternatively comprise depositing any other suitable insulating material by any suitable method at another surface of the heating region. In one variation, Block S260 comprises depositing a low temperature oxide by chemical vapor deposition (e.g., plasma-enhanced chemical vapor deposition) to form the second insulating layer, and in other variations, Block S260 can comprise fluid deposition of an insulating material (e.g., inkjet printing or casting of an electrically insulating polymer, softbaking/hardbaking of a deposited polymer, etc.) onto desired portions of the heating region.

In variations of the method 200 comprising Block S260, the method 200 can also further comprise Block S265, which recites removing material of the second insulating layer to produce a set of connection points. Block S265 functions to provide access to the heating region between insulation regions, such that the heating region can be electrically connected to the electronics substrate. The set of connection points can be defined using a material removal method including any one or more of: etching (e.g., lithography, laser etching), machining (e.g., drilling), and any other suitable method of material removal. In one such variation of Block S265, the set of connection points can be defined photolithographically using a positive resist process in a manner similar to that used in a variation of Block S230. In one example of this variation, the second insulating layer can be covered with positive photoresist and lithographically etched in exposed regions. In the example, the positive

photoresist can then be removed to reveal the connection points. In other variations, the connection points can be defined using any lithographic method, using positive and/or negative etching to form the heating pattern, and/or using any other suitable method. Upon definition of the connection points, the second insulating layer can further be etched (e.g., using buffered hydrofluoric acid) as an additional surface treatment. Alternative variations of Block S265 can include additionally or alternatively removing material from the first insulating surface of Block S210 to form any subset of the set of connection points.

As shown in FIG. 10, the method 200 can further include Block S270, which recites: coupling a non-reflective coating to at least one heater-sensor die of the set of heater-sensor dies. Block S270 functions to process at least a subset of the set of heater-sensor dies 110 so that they do not interfere with light transmitted to photodetectors of an optical subsystem opposed (e.g., directly opposed, in opposition) to the set of heater-sensor dies 110 (e.g., light emitted from the set of biological samples, light transmitted through filters of an optical subsystem), especially in configurations wherein a set of detection chambers is configured between the set of heater-sensor dies and optical elements of an optical subsystem. The non-reflective coating is preferably coupled identically to all heater-sensor dies of the set of heater sensor dies; however, the non-reflective coating can alternatively be coupled non-identically to one or more heater-sensor dies of the set of heater-sensor dies. As such, in variations, one or more subsets of the set of heater-sensor dies can be coupled to non-reflective coatings in a manner that provides different light reflection properties for the subset(s) of the set of heater-sensor dies.

In Block S270, the non-reflective coating is preferably a material layer that is applied superficial to at least one of the first insulating layer and the second insulating layer processed in variations of Blocks S210 and S260, respectively. In one example, the non-reflective coating processed in Block S270 can comprise a high-temperature paint (e.g., dark paint, flat paint) that functions to absorb and/or diffuse light from an opposing optical subsystem, while facilitating heat transfer to a set of detection chambers in thermal communication with the set of heater-sensor dies. In this example, the high-temperature paint can be applied by any one or more of: brushing, spraying, dipping, printing, and any other suitable method of coupling the high-temperature paint to one or more surfaces of at least a subset of the set of heater-sensor dies. However, the non-reflective coating can alternatively be processed simultaneously with or can comprise one or more of the first insulating layer and the second insulating layer processed in variations of Blocks S210 and S260, respectively. For instance, one or more of the first and the second insulating layer can include a modified oxide layer that has low-reflectivity, thus preventing interference caused by light reflected from the set of heater-sensor dies. In some extreme variations, however, mitigation of interference due to reflected light from the set of heater-sensor dies can be produced by configuring the set of heater-sensor dies to be in misalignment with photodetectors of the optical subsystem, such that reflection does not interfere with light transmitted to the photodetectors of the optical subsystem, in modified versions of Block S270. For instance, the set of heater-sensor dies can be configured to heat a set of detection chambers from a first direction, and the optical subsystem can be configured to receive light from the set of detection chambers from a second direction (e.g., a direction non-parallel to the first direction), such that reflection from the set of heater-sensor dies 110 does not

US 10,226,771 B2

17

cause interference. In still other variations of Block S270, the set of heater-sensor dies can be processed with any other suitable elements (e.g., coatings, layers, etc.) and/or be configured in any other suitable manner that eliminates, prevents, or mitigates reflection from the set of heater-sensor dies from interfering with light transmitted to photodetectors of an optical subsystem in opposition to the set of heater-sensor dies 110.

The method 200 can further comprise any other suitable block, such as calibrating sensing elements of the thermocycling system S280. In an example of Block S270, the set of heater-sensor dies coupled to the electronics substrate can be installed in thermal chamber to calibrate the sensing elements (i.e., RTDs) of the set of heater-sensor dies. In the example, the electronics substrate and a first connector end of a calibration system was placed in a thermal chamber and a second connector end of the calibration system was attached to an array of contacts outside the thermal chamber. The thermal chamber was allowed to equilibrate in stages at a series of temperatures spanning the expected dynamic range of the RTDs, from 30 C to 100 C in four stages. The RTD resistance values were read out at the various equilibrated temperatures, and fit a Callendar-Van Dusen equation. The calibration of the example of Block S270 yielded the coefficients used to convert the sensing element resistance values to temperature values, in order to calibrate the sensing elements of the system.

The system 100 and/or method 200 of the preferred embodiment and variations thereof can be embodied and/or implemented at least in part as a machine configured to receive a computer-readable medium storing computer-readable instructions. The instructions are preferably executed by computer-executable components preferably integrated with the system 300 and one or more portions of the processor 350. The computer-readable medium can be stored on any suitable computer-readable media such as RAMs, ROMs, flash memory, EEPROMs, optical devices (CD or DVD), hard drives, floppy drives, or any suitable device. The computer-executable component is preferably a general or application specific processor, but any suitable dedicated hardware or hardware/firmware combination device can alternatively or additionally execute the instructions.

The FIGURES illustrate the architecture, functionality and operation of possible implementations of methods according to preferred embodiments, example configurations, and variations thereof. In this regard, each block in the flowchart or block diagrams may represent a module, segment, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that, in some alternative implementations, the functions noted in the block can occur out of the order noted in the FIGURES. For example, two blocks shown in succession may, in fact, be executed substantially concurrently, or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved. It will also be noted that each block of the block diagrams and/or flowchart illustration, and combinations of blocks in the block diagrams and/or flowchart illustration, can be implemented by special purpose hardware-based systems that perform the specified functions or acts, or combinations of special purpose hardware and computer instructions.

As a person skilled in the art will recognize from the previous detailed description and from the figures and claims, modifications and changes can be made to the

18

preferred embodiments of the invention without departing from the scope of this invention defined in the following claims.

We claim:

1. A thermocycling system comprising:

a set of heater-sensor dies, each heater-sensor die in the set of heater-sensor dies comprising a heating element and a sensing element and comprising 1) a first surface that interfaces with a detection chamber during operation and 2) a second surface, inferior to the first surface, including a connection point;

an electronics substrate, comprising a first substrate surface coupled to the second surface of each of the set of heater-sensor dies, a set of apertures longitudinally spaced across the electronics substrate and providing access through the electronics substrate to the set of heater-sensor dies, a second substrate surface inferior to the first substrate surface, and a set of substrate connection points;

a set of conductors coupled between the connection points the set of heater-sensor dies and the set of substrate connection points by way of the set of apertures; and
a set of heat-sink supports operable to dissipate heat generated by the set of heater-sensor dies, wherein a base surface of each of the set of heat-sink supports is coupled to an elastic element that transmits a biasing force through the electronics substrate, thereby maintaining thermal communication between the set of heater-sensor dies and a set of detection chambers during operation of the system.

2. The thermocycling system of claim 1, wherein the electronics substrate is a flexible electronics substrate.

3. The thermocycling system of claim 1, wherein each of the set of heater-sensor dies includes a coating, proximal the heating surface, operable to mitigate reflection of light from the heating surface toward photodetectors of an optical subsystem opposed to the heating surface during operation of the thermocycling system.

4. The thermocycling system of claim 1, wherein the electronics substrate further includes a set of recesses, each recess of the set of recesses contiguous with at least one aperture of the set of apertures.

5. The thermocycling system of claim 4, wherein each recess of the set of recesses is configured to receive a heater-sensor die of the set of heater-sensor dies.

6. The thermocycling system of claim 4, wherein each heater sensor-die of the set of heater-sensor dies is coupled to the first substrate surface, and the set of substrate connection points is defined between the first substrate surface and the second substrate surface proximal to the set of recesses.

7. The thermocycling system of claim 1, wherein the set of substrate connection points comprises substrate connection points located at the first substrate surface, and wherein the set of substrate connection points couples the heating element and the sensing element of each of the set of heater-sensor dies to a controller.

8. The thermocycling system of claim 1, wherein the set of substrate connection points comprises substrate connection points located at internal surfaces of the set of apertures, and wherein the set of substrate connection points couples the heating element and the sensing element of each of the set of heater-sensor dies to a controller.

9. The thermocycling system of claim 1, wherein the set of substrate connection points comprises substrate connection points located at the second substrate surface, and wherein the set of substrate connection points couples the

US 10,226,771 B2

19

heating element and the sensing element of each of the set of heater-sensor dies to a controller.

10. The thermocycling system of claim 9, wherein the set of substrate connection points is distributed at regions of the second substrate surface between adjacent apertures of the set of apertures of the electronics substrate. 5

11. The thermocycling system of claim 1, wherein each of the set of heat-sink supports is coupled to at least one of 1) the set of heater-sensor dies, through the set of apertures, and 2) the second substrate surface of the electronics substrate. 10

12. The thermocycling system of claim 1, wherein at least one heat-sink supports of the set of heat-sink supports includes an integrated cooling element.

* * * * *

20

EXHIBIT 51



US010239060B2

(12) **United States Patent**
Brahmasandra et al.

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(45) **Date of Patent:** ***Mar. 26, 2019**

(54) **THERMOCYCLING SYSTEM,
COMPOSITION, AND MICROFABRICATION
METHOD**

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(*) Notice: Subject to any disclaimer, the term of this
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U.S.C. 154(b) by 101 days.

This patent is subject to a terminal dis-
claimer.

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B01L 1/00 (2006.01)

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(52) **U.S. Cl.**

CPC **B01L 7/52** (2013.01); **C23C 14/025**
(2013.01); **C23C 14/165** (2013.01); **C23C**
14/24 (2013.01); **C23C 14/34** (2013.01); **B01L**
2200/12 (2013.01); **B01L 2200/147** (2013.01);
B01L 2300/0819 (2013.01); **B01L 2300/0887**
(2013.01);

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(58) **Field of Classification Search**

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2300/1827; **B01L 3/502723**; **B01L 7/52**
See application file for complete search history.

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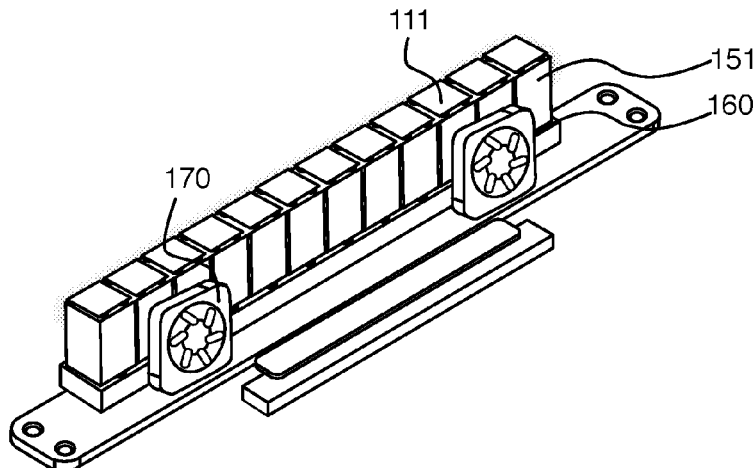
Primary Examiner — Nathan A Bowers

(74) *Attorney, Agent, or Firm* — Jeffrey Schox

(57) **ABSTRACT**

A system and method of manufacture for the system, comprising a set of heater-sensor dies, each heater-sensor die comprising an assembly including a first insulating layer, a heating region comprising an adhesion material layer coupled to the first insulating layer and a noble material layer, and a second insulating layer coupled to the heating region and to the first insulating layer through a pattern of voids in the heating region, wherein the pattern of voids in heating region defines a coarse pattern associated with a heating element of the heating region and a fine pattern, integrated into the coarse pattern and associated with a sensing element of the heating region; an electronics substrate configured to couple heating elements and sensing elements of the set of heater-sensor dies to a controller; and a set of elastic elements configured to bias each of the set of heater-sensor dies against a detection chamber.

20 Claims, 9 Drawing Sheets



US 10,239,060 B2Page 2

Related U.S. Application Data

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C23C 14/02 (2006.01)
C23C 14/16 (2006.01)
C23C 14/24 (2006.01)
C23C 14/34 (2006.01)

(52) **U.S. Cl.**

CPC ... *B01L 2300/123* (2013.01); *B01L 2300/168*
(2013.01); *B01L 2300/1827* (2013.01); *B01L*
2300/1883 (2013.01)

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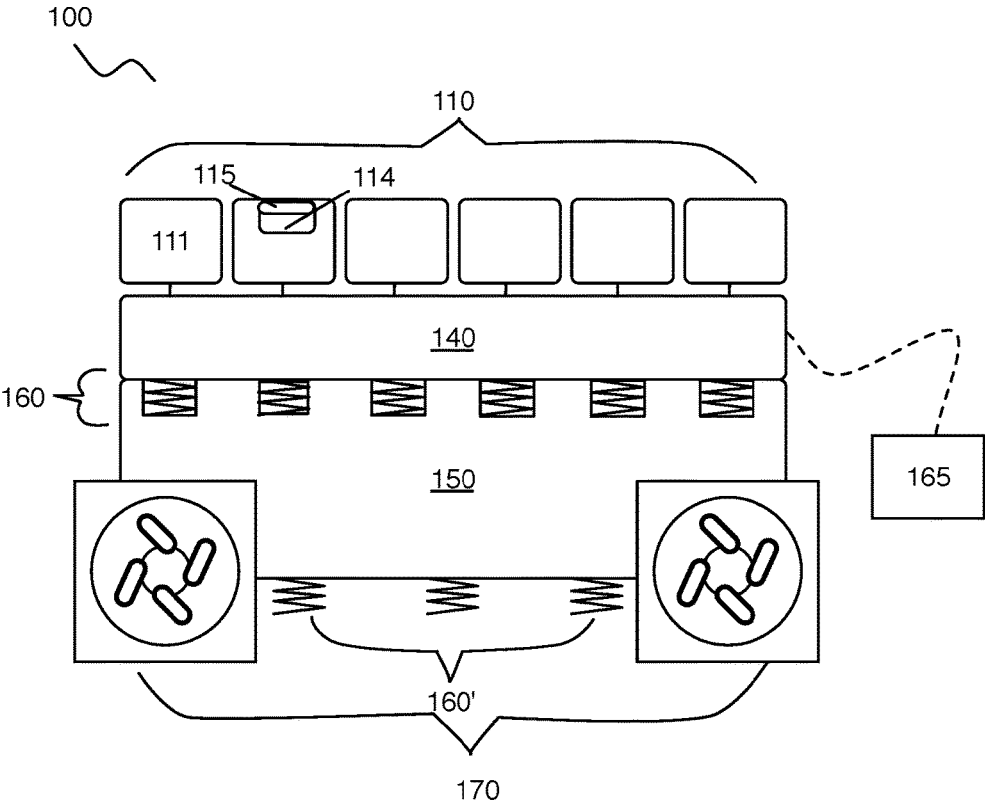


FIGURE 1A

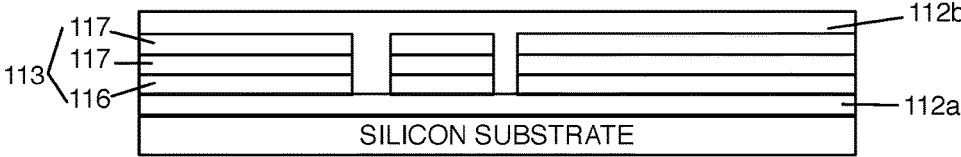
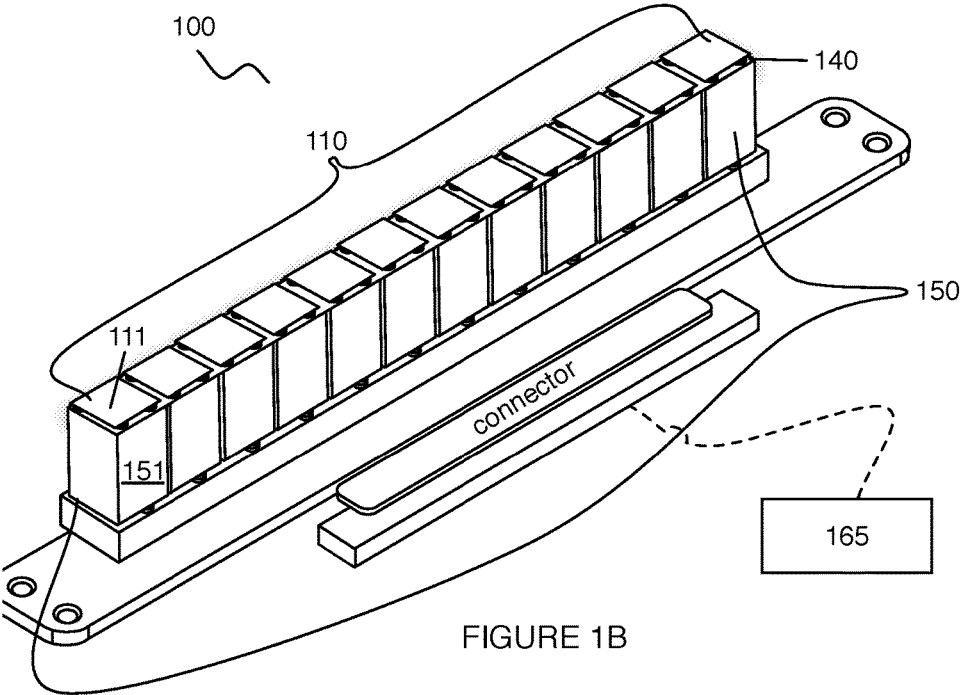
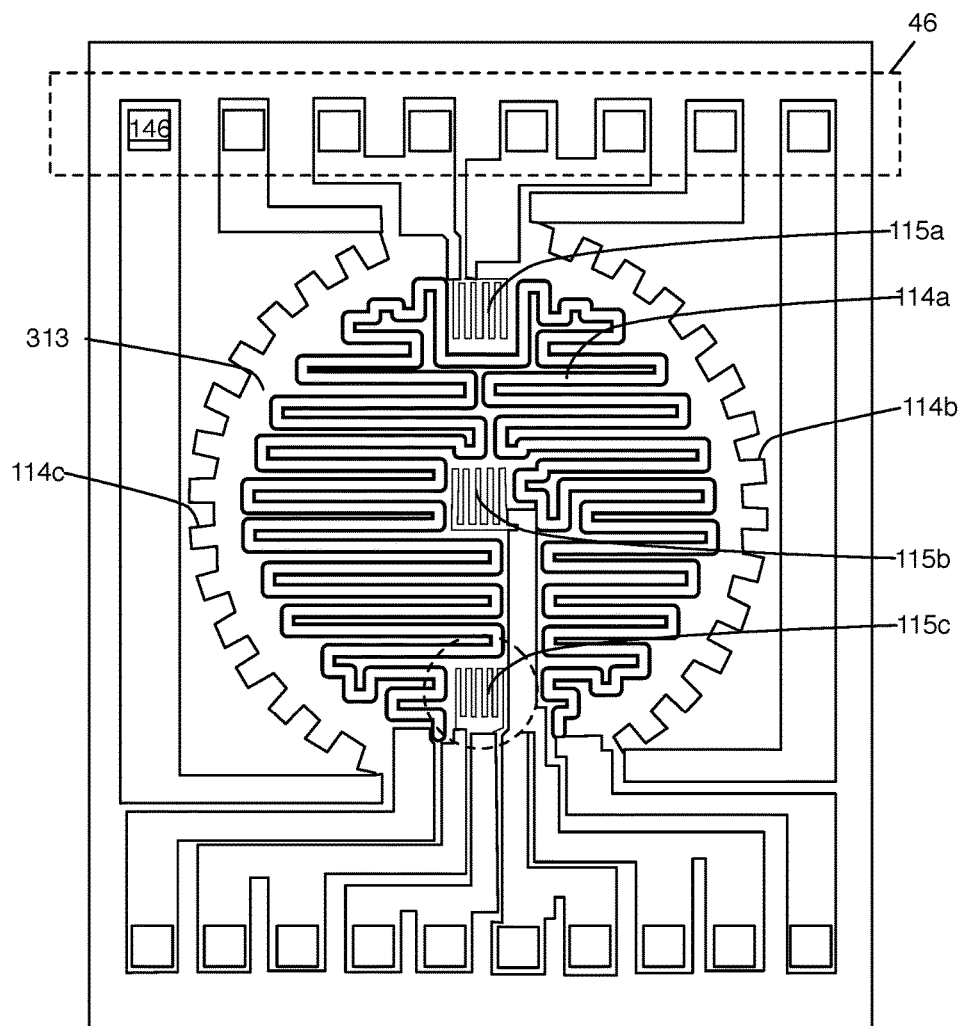


FIGURE 2



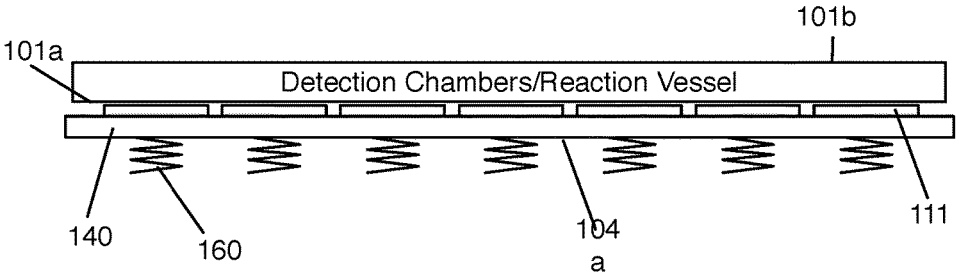


FIGURE 4A

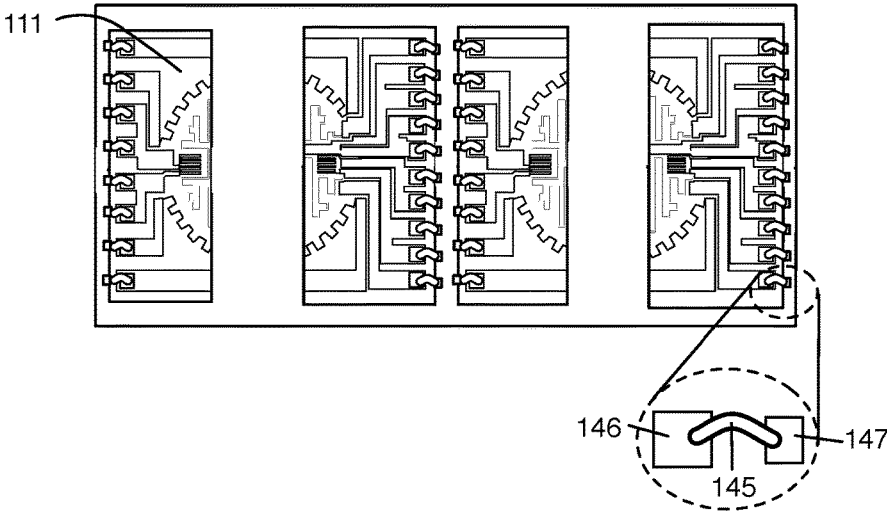


FIGURE 4B

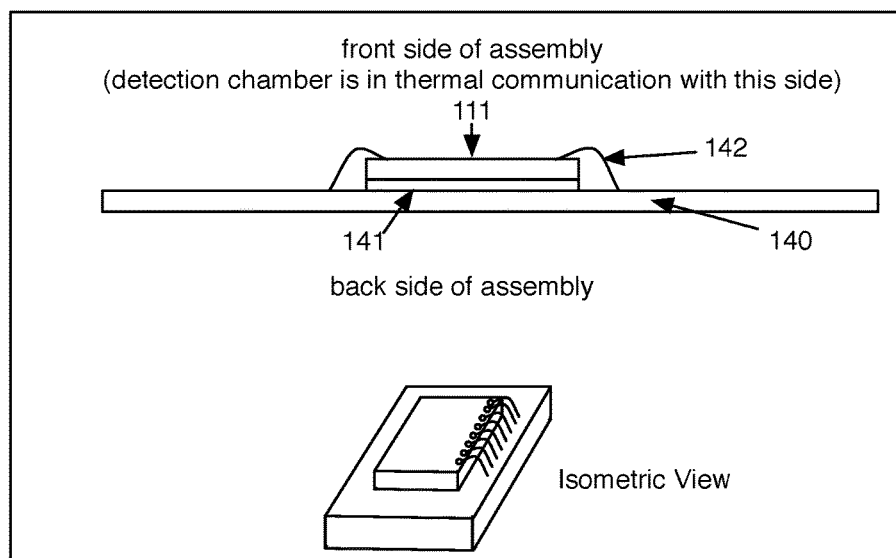


FIGURE 5A

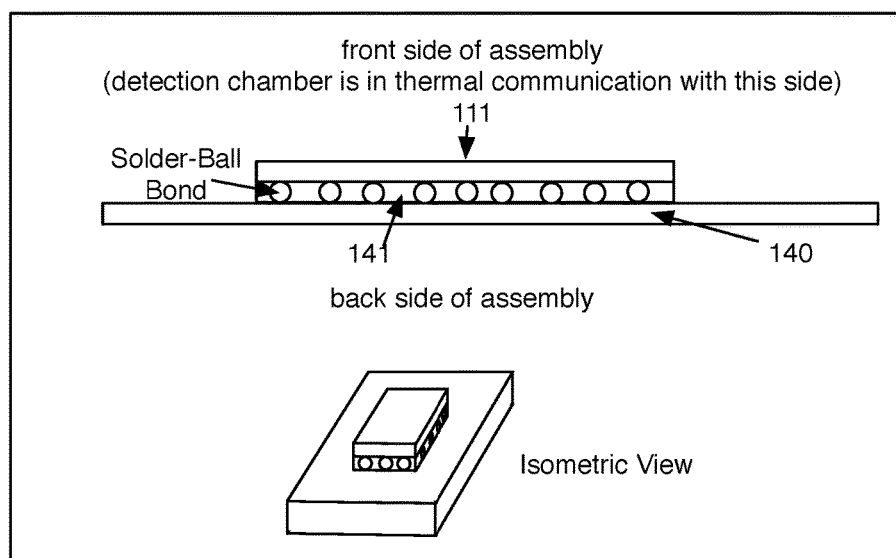


FIGURE 5B

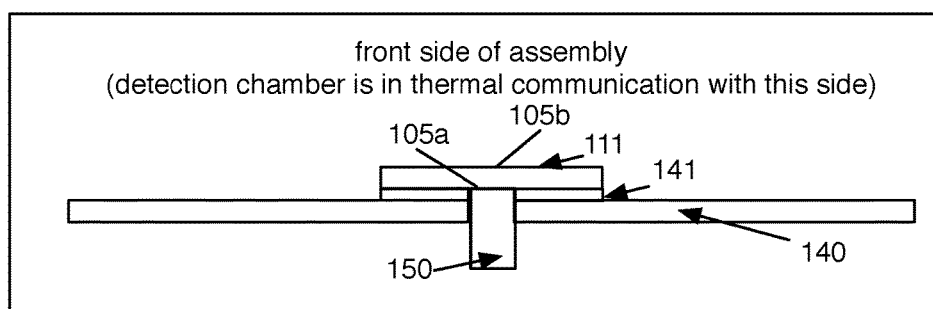


FIGURE 6A

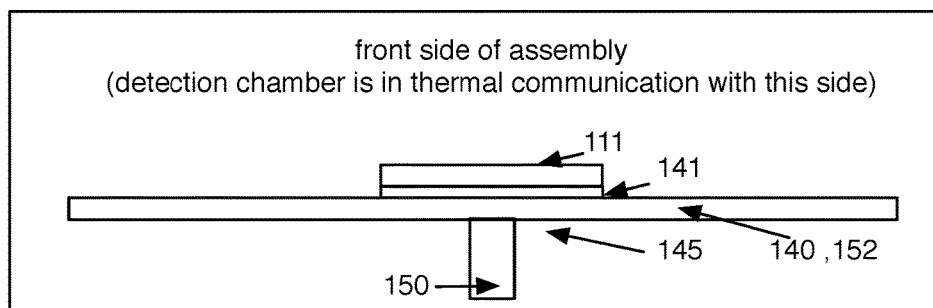
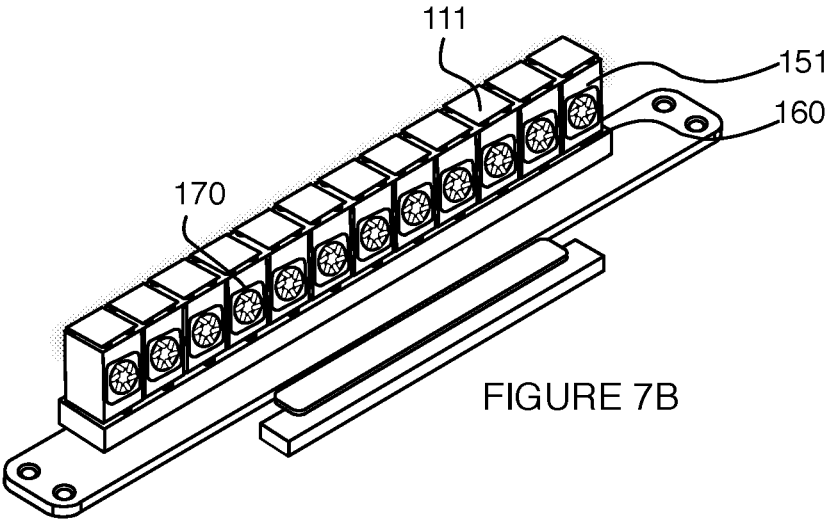
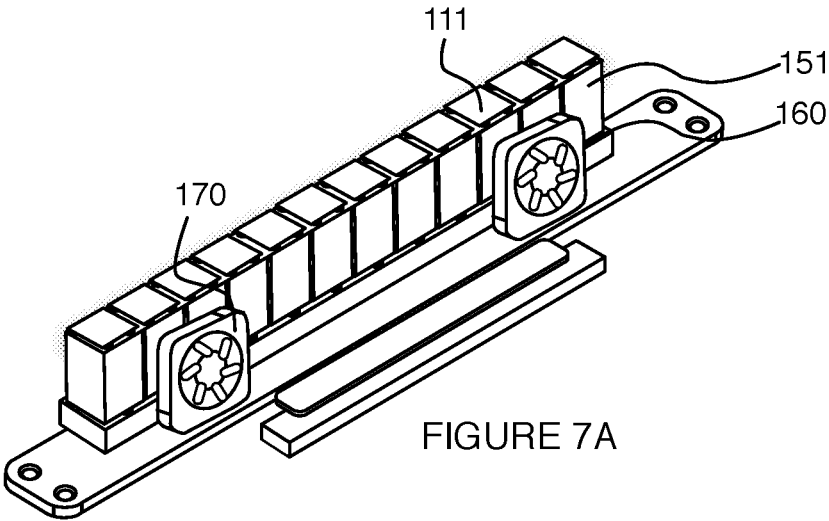


FIGURE 6B



U.S. Patent

Mar. 26, 2019

Sheet 8 of 9

US 10,239,060 B2

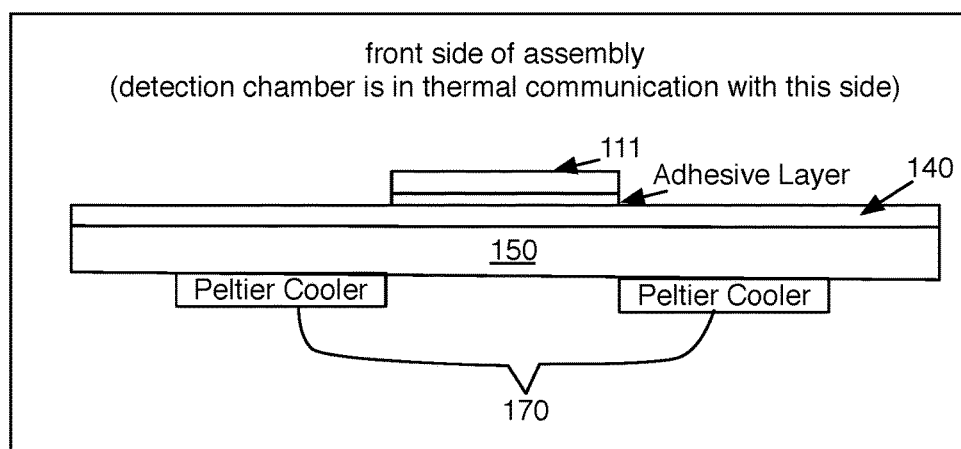


FIGURE 7C

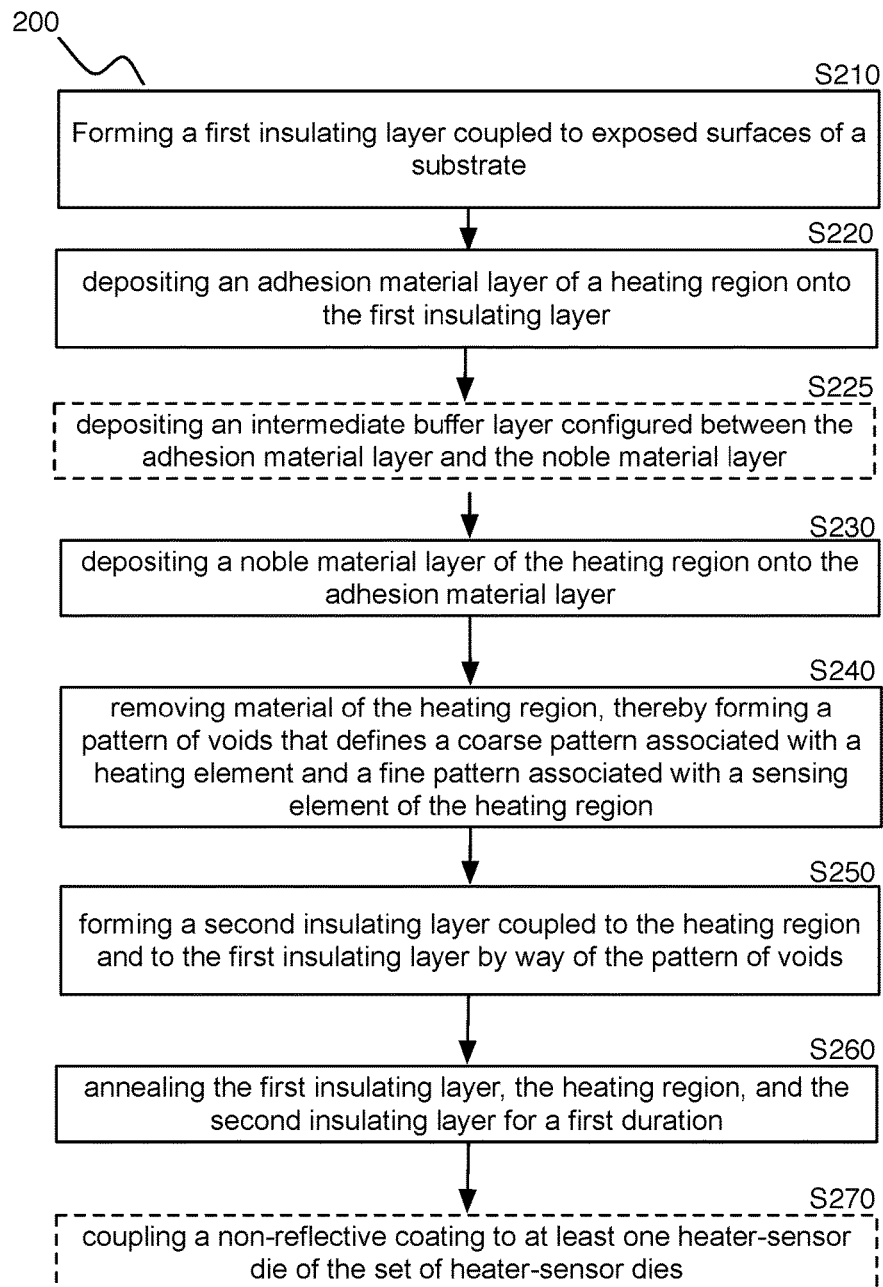


FIGURE 8

US 10,239,060 B2

1

THERMOCYCLING SYSTEM, COMPOSITION, AND MICROFABRICATION METHOD

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 14/487,808, filed 16 Sep. 2014 which claims the benefit of U.S. Provisional Application Ser. No. 61/879,513 filed 18 Sep. 2013, which is incorporated in its entirety herein by this reference.

TECHNICAL FIELD

This invention relates generally to the molecular diagnostics field, and more specifically to an improved sample thermocycling system and fabrication process thereof.

BACKGROUND

Molecular diagnostics is a clinical laboratory discipline that has developed rapidly during the last 25 years. It originated from basic biochemistry and molecular biology research procedures, but now has become an independent discipline focused on routine analysis of nucleic acids (NA), including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for diagnostic use in healthcare and other fields involving analysis of nucleic acids. Molecular diagnostic analysis of biological samples can include the detection of one or more nucleic acid materials present in the specimen. The particular analysis performed may be qualitative and/or quantitative. Methods of analysis typically involve isolation, purification, and amplification of nucleic acid materials, and polymerase chain reaction (PCR) is a common technique used to amplify nucleic acids. Often, a nucleic acid sample to be analyzed is obtained in insufficient quantity, quality, and/or purity, hindering a robust implementation of a diagnostic technique. Current sample processing methods and molecular diagnostic techniques are often labor/time intensive, low throughput, and expensive, and systems of analysis are insufficient.

A rapid and efficient thermocycling system that can reliably thermocycle reagents used for processing of nucleic acids can significantly improve the efficiency and effective implementation of molecular diagnostic techniques, such as realtime polymerase chain reaction (RT-PCR). Microfabrication techniques can produce such thermocycling systems comprising precision heaters with low thermal masses and with well-coupled temperature sensors. However, challenges are inherent in ensuring that the microfabrication and assembly processes utilized to fabricate thermal cycling elements are extremely robust and reliable.

Due to these challenges and deficiencies of current molecular diagnostic systems and methods, there is thus a need for an improved sample thermocycling system and fabrication process thereof. This invention provides such a system and fabrication process.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B depict embodiments of a thermocycling system;

FIG. 2 depicts an example schematic of a heater-sensor die during fabrication in an example of a thermocycling system;

2

FIG. 3 depicts an example of heating and sensing elements in an example of a thermocycling system;

FIGS. 4A and 4B depict examples of additional elements of an embodiment of a thermocycling system;

FIGS. 5A and 5B depict variations of configurations of elements in an embodiment of a thermocycling system;

FIGS. 6A and 6B depict variations of configurations of elements in an embodiment of a thermocycling system;

FIGS. 7A-7C depict variations in configurations of additional elements in an embodiment of a thermocycling system; and

FIG. 8 depicts a schematic of an embodiment of a method for manufacturing a thermocycling system.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description of the preferred embodiments of the invention is not intended to limit the invention to these preferred embodiments, but rather to enable any person skilled in the art to make and use this invention.

1. System

As shown in FIGS. 1A and 1B, an embodiment of a system **100** for thermocycling biological samples within detection chambers comprises: a set of heater-sensor dies **110**; an electronics substrate **140** that couple the set of heater-sensor dies to a controller; a set of heat sink supports **150** coupled to at least one of the electronics substrate and the set of heater-sensor dies; and a set of elastic elements **160** coupled to the electronics substrate and configured to bias each of the set of heater-sensor dies against a detection chamber. In some embodiments, the system **100** further comprises a controller **165** and/or a cooling subsystem **170** configured to actively cool the system **100**.

The system **100** functions to enable rapid thermal cycling of samples while providing uniform heating and preventing signal drift. In specific applications, the system **100** can be used to rapidly and controllably thermocycle nucleic acid samples during performance of molecular diagnostic amplification techniques (e.g., PCR, RT-PCR), signal amplification techniques (e.g., bDNA, hybrid capture), and analytical techniques (e.g., gel electrophoresis, mass spectrometry). The system **100** can also provide rapid thermocycling without significant power requirements, ensure a closer correlation between the actual heating temperature and the temperature set-point by implementing an integrated heater-sensor die, and controllably and individually heat small sample volumes (e.g., picoliters, nanoliters) based upon a microfabrication technique that also enables mass production of the system **100**. Furthermore, the system **100** implements a priori predictions of electrical resistance values of thin film combinations of the set of heater-sensor dies **110**, and accounts for and/or prevents signal drift to maintain controlled sample heating. In some variations, the system **100** can be integrated into a molecular diagnostic system, such as that described in U.S. Pub. No. 2013/0210015, entitled "System and Method for Processing and Detecting Nucleic Acids", and filed on 13 Feb. 2013; however, the system **100** can additionally or alternatively be used with any other suitable system for processing biological or non-biological samples.

1.1 Heater-Sensor Dies

The set of heater-sensor dies **110** functions to controllably heat individual sample volumes. Preferably, each heater-sensor die **111** is a thin-film die that can be deposited onto another substrate (e.g., silicon, glass substrate) that can be packaged onto an electronics substrate **140** (e.g., printed

US 10,239,060 B2

3

circuit board, PCB); however, each heater-sensor die **111** can alternatively comprise any suitable geometry and/or configuration that enables controlled, uniform, and rapid heating of a detection chamber in thermal communication with the heater-sensor die **111**. In some embodiments, the detection chambers can be those described in U.S. Pub. No. 2013/0209326, entitled "Microfluidic Cartridge for Processing and Detecting Nucleic Acids" and filed on 13 Feb. 2013, which is herein incorporated in its entirety by this reference; however, the detection chambers can alternatively be any other suitable container for processing a biological sample. Preferably, each heater-sensor die **111** is characterized by a small profile (e.g., <10 mm dimension), which ensures that the heater-sensor die **111** is able to thermocycle rapidly; however, a heater-sensor die **111** can alternatively be characterized by any suitable profile in order to meet any other thermocycling requirement. Additionally, each heater-sensor die **111** in the set of heater-sensor dies **110** is preferably configured to conform to a detection chamber (e.g., sample tube, sample container, sample heating zone of a cartridge for processing samples) configured to contain a sample during heating; however, a heater-sensor die **111** in the set of heater-sensor dies **110** can alternatively not conform to a detection chamber for processing of a biological sample.

Preferably, each heater-sensor die **111** in the set of heater sensor dies **110** comprises an assembly including: a first insulating layer **112a** that functions to provide an insulating barrier to isolate the heaters and sensors and a heating region **113** that functions to provide uniform sample heating. Some variations can further include a second insulating layer **112b** that functions to provide an additional insulating barrier that opposes the first insulating layer **112a**. The insulating layer(s) **112a**, **112b** are preferably electrically insulating, but can additionally be thermally insulating in variations where localized heating is also desired. Furthermore, each heater-sensor die **111** preferably comprises two insulating layers **112a**, **112b** that are configured to "sandwich" the heating region **113**, thus isolating the heating region **113** at least at two surfaces of the heating region **113**; however, each heater-sensor die **111** can alternatively comprise any suitable number of insulating layers **112** arranged relative to the heating region **113** in any suitable manner. In one variation, as described in further detail below, the heating region **113** can include a pattern of voids that defines elements of the heating region, and a second insulating layer **112b** can be configured to couple to the heating region **113** and to the first insulating layer **112a** through the pattern of voids in the heating region **113**, as shown in FIG. 2. The second insulating layer **112b** can, however, be configured to couple to the heating region **113** and/or to the first insulating layer **112a** in any other suitable manner.

The heating region **113** preferably comprises a heating element **114** with an integrated sensing element **115**, as shown in FIG. 3, and is composed of at least one metal or metal alloy, with configurations described in further detail below. Furthermore, the heating region **113** preferably defines a pattern **313** of voids having geometric features (e.g., width, thickness, length, spacing) that facilitate uniform heating and provide desired heating and sensing characteristics (e.g., resistance characteristics). In some variations, the pattern **313** can comprise any one or more of: linear segments, non-linear segments, boustrophedonic segments, continuous segments, non-continuous segments, and any other suitable segment(s) having any other suitable geometry (i.e., width, depth, height, length, path, etc.). The pattern **313** can be symmetric about any suitable reference (e.g., reference line, reference plane, etc.), or can alternatively

4

be non-symmetric. Furthermore, in some variations, the pattern **313** can define a global morphology (e.g., circular footprint, ellipsoidal footprint, polygonal footprint, etc.) at a first scale (e.g., macroscopic scale) but have a local morphology at a second scale (e.g., microscopic scale), wherein the local morphology provides desired characteristics (e.g., resistance characteristics) that are attributed to elements (e.g., heating elements **114**, sensing elements **115**) of the heating region **113**. As such, the global morphology can provide conformation (e.g., in shape) between the heating region **113** and a detection chamber coupled to or corresponding to a heater-sensor die **111**, and the local morphology can provide uniform heating and/or accurate sensing of heating parameters by utilizing structural features having a smaller governing dimension.

In a specific example, as shown in FIG. 3, the heating elements **114a**, **114b**, **114c** with integrated sensing elements **115a**, **115b**, **115c** are defined by a pattern **313** with a global morphology characterized by a circular footprint, but a local morphology of structures characterized by a continuous boustrophedonic arrangement, wherein both the global morphology and local morphology were designed according to theory and fabricated as described in further detail below. In the specific example, the sensing element **115** is configured to be centrally located within the global morphology of the heating element **114**. However, in alternative variations, the heating region **113** can alternatively not comprise an integrated sensing element **115**, can comprise any suitable number of heating elements **114**/sensing elements **115**, and/or can be composed of any other suitable material.

Furthermore, during processing, each heater-sensor die **111** in the set of heater-sensor dies **110** is preferably annealed, as described in further detail below, to improve adhesion and settling of the heater-sensor materials (e.g., on a silicon substrate). The annealing can comprise a single stage of annealing or multiple stages of annealing, and can additionally or alternatively comprise different conditions (e.g., temperatures, durations, environmental conditions) during the annealing stage(s). To exemplify an effect that changes in resistance behavior can have upon heating and sensing function, a ~1-2 Ω deviation in resistance can cause significant deviations (e.g., 5-10° C.) between intended and actual temperatures attained by a heater-sensor die **111**. Such deviations in temperature can lead to unwanted variability in molecular diagnostic technique results and/or unreliable data (e.g., false positives, false negatives, indeterminate results). Even further, temperature swings greater than 10° C. can result in permanent damage to the thermocycling system **100** and/or to a sample being processed using the system **100**. Given the importance of maintaining the calibration of the sensing elements **115** over long periods of time, it is preferable to ensure that minimal dynamics occur in the materials of the heater-sensor dies **111** after fabrication is complete. Since a majority of changes in resistance (e.g., drift) come from thermal reactions, settling, diffusion, and improved adhesion, annealing can accelerate the processes that lead to changes in resistance, thus producing heater-sensor dies **111** with stable resistance behavior (e.g., stable for 3-5 years) by driving dynamic processes toward an equilibrium state (or other stable state) prior to use of the heater-sensor dies **111** in sample-processing applications. Preferably, annealing thus produces stable resistances that are substantially low (in order to enable driving at low voltages) and have limited variability, indicating that underlying material dynamics have reached equilibrium. Alternatively, during processing, each heater-sensor die **111** in the set of heater-sensor dies **110** may not be annealed, can

US 10,239,060 B2

5

comprise elements that limit temperature swings, and/or may undergo any other suitable process to produce stable resistance behavior in any other suitable manner.

In one variation, as shown in FIG. 2, the insulating layers **112a**, **112b** of the heater-sensor die **111** are composed of an oxide (e.g., silicon oxide), and the heating region **113** comprises an adhesion material layer **116** coupled to the first insulating layer **112a** and configured to promote adhesion of additional deposited layers (e.g., a noble material layer **117**) of the heating region **113**, and at least one noble material layer **117** coupled to the adhesion material layer **116** and configured to reduce or prevent signal drift that can result due to diffusion of the adhesion layer **116**. In this variation, the first insulating layer **112a** is grown or deposited uniformly on the surface of a silicon wafer (e.g., by thermal oxidation), the material layers **116**, **117** of the heating region **113** are deposited upon the first insulating layer **112a** (e.g., by evaporation, by sputtering, etc.), the heating region **113** is etched to define the heater/sensor pattern **313** of the heating element **114** and the sensing element **115** (e.g., by lithography using a positive resist, by lithography using a negative resist), and a second insulating layer **112b** is deposited on the heating region **113** (e.g., by chemical vapor deposition) to insulate the heating region **113** between two insulating oxide layers. Preferably, the adhesion materials used in the adhesion material layer(s) **116** comprise materials that are oxygen-active to react (e.g., chemically react) with an oxide surface (e.g., of an insulating layer **112a**, **112b**). In examples, the adhesion material layer **116** can be composed of any one or more of: chromium, titanium, niobium, vanadium and any combination or alloy thereof. In examples, the noble material layer can be composed of any one or more of: gold, platinum, tungsten, palladium, and any combination or alloy thereof. Furthermore, variations of the examples can include any suitable number of adhesion material layer(s) **116** and/or noble material layer(s) **117** coupled between the insulating layer(s) **112a**, **112b**, wherein each layer **116**, **117** can have any other suitable thickness.

In a first specific example of this variation, as shown in FIG. 3, a heater-sensor die **111** is configured to uniformly heat a circular region having a diameter of 5 mm, the heater-sensor die **111** has a footprint spanning a region of ~8.6 mm×7 mm, and the heating region **113** of the heater-sensor die **111** defines three heating elements **114a**, **114b**, **114c**: a central circular heating element **114a** and two circumferential heating elements **114b**, **114c** configured to form a boundary with a serpentine-shaped pattern about the central circular heating element **114a**. In the first specific example, the two circumferential heating elements **114b**, **114c** each form a semicircular perimeter about the central circular heating element **114a**. The heater-sensor die **111** in the first specific example further comprises three integrated sensing elements (i.e., resistance temperature sensors, RTDs) distributed at three locations within the 5 mm circular region. In the first specific example, the heating elements **114** comprise a 50 nm chromium adhesion material layer **116** and a 200 nm gold noble material layer **117** and were etched away in a boustrophedonic pattern to define the pattern of voids **313**, designed using a layout editor (e.g., Mentor Graphics™ or L-Edit™), to form the heating elements **114** and the sensing elements **115**. The heating elements **114a**, **114b**, **114c** are defined by coarse patterning in the first specific example, and the sensing elements **115** are integrated with the heating elements **114a**, **114b**, and **114c** and defined by fine patterning, as shown in FIGS. 3A and 3B. In the first specific example, the heating elements **114** are characterized with resistances in the range of

6

40-100Ω and the accompanying sensing elements **115** (i.e., RTDs) are characterized with resistances in the range of 200-250Ω prior to annealing. With annealing at 400 F in an inert N₂ atmosphere, the resistances for both the heating elements **114** and the sensing elements **115** increased 400-600% after 1 hour, and increased up to 800% with increasing anneal times.

In other embodiments of the heater-sensor dies **111**, the heating element(s) **114** and/or the sensing element(s) **115** can comprise other combinations of adhesion material layer(s) **116** and/or noble material layer(s) **117** in addition to or alternative to chromium and gold, including any one or more of: titanium (adhesion), platinum (noble), tungsten (noble), and any combination or alloy thereof. In particular, chromium and titanium can serve as preferable adhesion materials for an active noble layer being coupled to the adhesion material layer **116**, due to attributes that enable them to react with oxide materials. The combination of chromium and gold additionally can undergo processing by a lift-off method or an etching process to form a heating element **114** and/or a sensing element **115**, as described in further detail in Section 2 below. While in some applications it can be difficult to etch platinum films due to platinum's non-reactivity, platinum has a preferable temperature coefficient of resistance (TCR) of ~0.00385 Ω/Ω/C that makes it stable and preferable for RTD fabrication. Platinum and/or titanium can even serve as an intermediate buffer layer **119**, configured between an adhesion material layer **116** and a noble material layer **117**, that can prevent diffusion from an adhesion material layer **116** into a noble material layer **117** and stabilize resistance behavior (e.g., electrical resistance dynamics). As such, variations of the heating region **113** can include any suitable number of intermediate buffer layers **119** that prevent diffusion between an adhesion material layer **116** into an adjacent noble material layer **117**.

In a second specific example, the heating elements **114** comprise a 50 nm chromium adhesion material layer **116**, an 100 nm platinum intermediate buffer layer **119** coupled to the adhesion material layer **116**, and a 300 nm gold noble material layer **117** coupled to the intermediate buffer layer **119** and were etched away in an identical pattern to that in the first specific example to form a surface including the heating and sensing elements **114**, **115**. Similar to the first specific example, the heating elements **114** of the second specific example are defined by coarse patterning, and the sensing elements **115** are defined by fine patterning and integrated into the coarse patterning of the heating elements **114**. Also similar to the first specific example, the heating region **113** is configured between two insulating layers **112a**, **112b** in the second specific example. In the second specific example, the heating elements **114** were characterized to have resistances of approximately 50Ω and the sensing elements were characterized to have resistances around 130Ω prior to annealing. After annealing at 400 F in an inert N₂ atmosphere, the resistances for both the heating elements **114** and the sensing elements increased 10-30% after 1 hour, with no substantial further change in resistance following additional annealing of 2 hours.

In a third specific example, the heating elements **114** comprise a 50 nm chromium adhesion material layer **116** and a 100 nm platinum noble material layer **117** coupled to the adhesion material layer **116** and were etched away in an identical pattern to that in the first specific example, to form a surface including the heating and sensing elements **114**, **115**. Similar to the first specific example, the heating elements **114** of the third specific example are defined by coarse patterning, and the sensing elements **115** are integrated with

the coarse patterning of the heating elements **114** and defined by fine patterning. Also similar to the first specific example, the heating region **113** is configured between two insulating layers **112a**, **112b** in the third specific example. In the third specific example, the heating elements **114** are characterized with resistances around 2Ω prior to annealing. After annealing at 400 F in an inert N_2 atmosphere, the resistances for the heating elements **114** decreased 7% after 1 hour, with no substantial change in resistance following additional annealing of 2 hours.

In a fourth specific example, the heating elements **114** comprise a 50 nm titanium/tungsten adhesion material layer **116** and a 440 nm gold noble material layer **117** and were etched away in an identical pattern to that in the first specific example, to form a surface including the heating and sensing elements **114**, **115**. Similar to the first specific example, the heating elements **114** are defined by coarse patterning, and the sensing elements **115** are integrated with the coarse patterning of the heating elements **114** and defined by fine patterning. Also similar to the first specific example, the heating region **113** is configured between two insulating layers **112a**, **112b** in the fourth specific example. In the fourth specific example, the heating elements **114** are characterized with resistances around 40Ω and the sensing elements **115** are characterized with resistances around 100Ω prior to annealing. After annealing at 400 F in an inert N_2 atmosphere, the resistances for both the heating elements **114** and the sensing elements **115** decreased 11-14% after 1 hour, with no substantial further change in resistance following additional annealing of 2 hours.

In the second, third, and fourth specific examples, analysis of the resistance values pre-annealing and post-annealing indicated that the compositions and processing of the respective thin film layers **112**, **116**, **117** significantly stabilize the resistance values and dynamics of resistance behavior of the heating and sensing elements **114**, **115** after annealing. The third specific example with chromium and platinum layers demonstrated substantially no variance from pre-anneal measurements of the electrical resistance after the second annealing step, and the fourth specific example with titanium, tungsten, and gold layers demonstrated a 11%-14% reduction in resistance following the first annealing step but then showed no additional variance after the second annealing step. No further changes in resistance following stages of annealing indicate that the dynamic behavior of the materials has been arrested or completed (e.g., has reached equilibrium). The second specific example with chromium, platinum, and gold layers demonstrated a slightly higher variance (~10%-30%) from pre-anneal resistance values indicating a higher level of dynamic behavior in these thin films, and further annealing was not completely sufficient to arrest the dynamics with this combination. However, the combination of different adhesion and noble materials with the intermediate buffer layer(s) **119** to reduce or eliminate diffusion between adhesion layers **116** and noble layers **117**, along with the annealing stage(s) produced positive effects with regard to stability of resistance behavior.

Other variations and examples of the heater-sensor dies **111** can comprise any suitable patterning configuration, any suitable arrangement of insulating layer(s) **112** and heating region(s) **113**, any suitable combination of adhesion, buffer, and/or noble layers **116**, **119**, **117**, and/or any suitable annealing or other process that facilitates stabilization of resistance behavior in the set of heater-sensor dies **110**.

1.1.1 Heater-Sensor Theory

In the specific examples described above, the microfabricated heater-sensor dies **111** of the set of heater-sensor dies

110 are configured in a manner that produces rapid thermocycling for a given level of power input. Design of the pattern **313** of heater-sensor dies **111** in the specific examples was performed using a layout editor (e.g., Mentor Graphics™ or L-Edit™), in order to generate the boustrophedonic structures of the local morphology of the heater elements **114**, within the bounds of the global circular morphology of the heater elements **114**. Estimates of the electrical resistance of both thin-film heating elements **114** and sensing elements **115** (e.g., RTDs) of the specific examples was performed based upon a determination of sheet resistances of the thin film layer(s) of the adhesion and noble layers **116**, **117** being deposited upon the insulating layer **112**, with thicknesses of the thin film layers ranging from 50 nm-500 nm. In the specific example, sheet resistances were calculated based on the resistivity of the thin film and the width, thickness, and length of the thin film regions of the adhesion and noble layers **116**, **117** being deposited. As such, in the specific examples, the layouts of the heating and sensing element **114**, **115** patterns are configured to obtain desired heater-sensor coverage on a region (e.g., a region proximal to a surface of a sample container) being thermally cycled. In the specific examples, the configuration of the heater-sensor dies **111** also ensures that electrical resistance values of the heater-sensor dies are in within a desirable range, from an energy-use standpoint (e.g., a range that enables a range of temperatures with a low input voltage).

Theoretically, the resistance(s) of the heating element(s) **114** are preferably in the range of 50-100 Ω to enable efficient heating of a substrate with an input voltage from 20-30 volts; however, the heating elements **114** can alternatively be characterized by other resistance ranges. A heating element **114** design characterized by a resistance between 50-100 Ω and that uses an input voltage of 20-30 volts provides a dissipation of 4-18 Watts of heat and is capable of rapidly heating a desired region of a thermally conductive substrate (e.g., silicon substrate). In addition, such a design parameters can produce a temperature across a 1 cm \times 1 cm substrate that equilibrates and produces a uniform temperature profile in a diffusion time of less than 1 second. Such a diffusion time thus enables well-controlled biochemical reactions, with regard to temperature uniformity and control.

Theoretically, the resistance(s) of the sensing element(s) **115** (e.g., RTD) are preferably in the range 200-300 Ω , based upon correlating a "change in resistance" (dependent upon TCR) against the temperature of the sensing element **115**. In one variation, the resistance behavior of an RTD can be characterized by the following equation:

$$R = R_o * (1 + \alpha * \Delta T + \beta * \Delta T^2)$$

where R=Resistance of an RTD at an unknown temperature (T); R_o =Resistance of temperature at a reference temperature T_o (e.g., room temperature); α and β =experimentally determined constants; and ΔT =Difference in temperature ($T - T_o$). Under this equation, the change in resistance (ΔR) for an RTD per degree change ($\Delta T=1$) is given by $\Delta R = (\alpha + \beta) * R_o$.

As such, for efficient temperature sensing as well as ensuring a detected temperature resolution of 0.1-0.2 C using RTDs, the resistance change per degree change in temperature is preferably greater than ~0.1 Ω . While higher changes in resistance can be desired, such higher changes in temperature, for a given RTD material or thin film combination, results only when the initial resistance (R_o) of the RTD is substantially high. However, having a substantially high initial resistance increases the risk of the RTD self-

heating during the resistance sensing process, thereby potentially causing additional noise/disturbances and inaccuracies in temperature measurement. The RTDs in a specific example of the system **100** were calibrated by measuring the resistance against 4-5 temperature points in the range the sensor is intended to be utilized. For biochemical assays, the typical range of temperatures is room temperature (25° C.) to ~100° C. By characterizing the resistance (R) at each of several temperatures (T) within a range of intended operation, one can obtain an experimental relationship for R vs. T.

1.2 Other System Elements

As shown in FIGS. 1, 4A-4B, and 7A-7C, the system **100** can further comprise an electronics substrate **140** configured to couple heating and sensing elements of the set of heater-sensor dies to a controller **165**, a set of heat-sink supports **150** configured to facilitate heat dissipation within the system **100**, a set of elastic elements **160** configured to bias the set of heater-sensor dies **110** against detection chambers for sample processing, and can additionally comprise the controller **165** and/or a cooling subsystem **170**.

The electronics substrate **140** is preferably coupled to the set of heater-sensor dies **110**, and functions to enable communication between heating elements **114** and sensing elements **115** of each heater-sensor die **111** in the set of heater-sensor dies **110** and a controller **165**. As such, the electronics substrate preferably enables communication of heat output commands from the controller **165** to the heating element(s) **114**, and communication of heating parameters (e.g., detected temperatures) sensed at the sensing element(s) **115** to the controller **165**. The electronics substrate **140** preferably comprises a printed circuit board (PCB), and in one variation is a flexible PCB, as shown in FIG. 4A, in order to facilitate contact between heater-sensor dies **111** in the set of heater-sensor dies **110** and detection chambers (e.g., reaction vessels, sample containers, etc.) for processing of a sample according to molecular diagnostic protocols. Alternatively, the PCB can be a substantially rigid PCB or any other suitable PCB.

Preferably, the set of heater-sensor dies **110** is coupled to the electronics substrate **140** in a manner that provides thermal and/or electrical isolation of each heater-sensor die **111** from the neighboring heater-sensor die(s) due to poor conductivity of the electronics substrate **140**. However, the electronics substrate **140** and the set of heater-sensor dies **110** can be configured in any alternative suitable manner that provides isolation of each heater-sensor die **111**. In some variations, each heater-sensor die **111** is coupled to the electronics substrate by an adhesive layer **141** and/or a wire bond **142**, as shown in FIG. 5A; however, each heater-sensor die **111** can alternatively or additionally be coupled to the electronics substrate **140** in any suitable manner (e.g., using solder bonds in flip-chip bonding), as shown in FIG. 5B. Furthermore, the system **100** can comprise any suitable number of electronics substrates.

The set of heat sink supports **150** is preferably coupled to at least one of the set of heater-sensor dies **110** and the electronics substrate **140** and functions to facilitate rapid thermocycling by dissipating heat from the set of heater-sensor dies **110** and/or the electronics substrate **140**. The set of heat sink supports **150** can further function to provide structural support for the set of heater-sensor dies **110**, such that the set of heater-sensor dies **110** is supported during compression (e.g., compression against a set of detection chambers) and/or tension. In the absence of heat sinking, the electronics substrate **140** and the surrounding environment can potentially retain too much heat, which compromises the cooling of the set of heater-sensor dies **110**. The set of heat

sink supports **150** can comprise multiple heat sink supports **151** configured to define any suitable number of contact locations, or can alternatively comprise a single heat sink support **151** configured to define any suitable number of contact locations. As shown in FIGS. 6A and 6B, the system **100** preferably couples to a detection chamber (e.g., reaction vessel, detection chamber) at a first side **101a** of the detection chamber, as shown in FIG. 4A, which can restrict heat dissipation from the first side **101a** of the system. Furthermore, the second side **101b** of the detection chamber is typically used for optical imaging for monitoring (e.g., realtime monitoring, delayed monitoring), and further limiting heat-sinking from the second side **101b**. Thus, it is preferable for the set of heat sink supports **150** to couple to the system **100** from a side of the system **100** that does not physically interfere with optical imaging apparatus interfacing with the system **100**. However, alternative configurations of the set of heat sink supports **150** can comprise coupling at any suitable side and/or any number of sides of the system **100**.

As shown in FIGS. 6A and 6B, the set of heat sink supports **150** can be configured in any of a number of variations. In a first variation, each heat sink support **151** can be directly placed against a first surface **105a** of heater-sensor die **111** opposing that of a second surface **105b** contacting a detection chamber, as shown in FIG. 6A. The first variation enables efficient transfer of heat out of the first surface **105a** of the heater-sensor die away from a respective detection chamber; however, excessive heat sinking can affect heating ramp rates. In a second variation, the system **100** comprises a thermally insulating assembly **152** between a heater-sensor die **111** and a corresponding heat sink support **151**, as shown in FIG. 6B. In the second variation, the electronics substrate **140** can serve as the thermally insulating assembly **152** and can be situated between the heater-sensor die **111** and a heat sink support **151**. Furthermore, in the second variation, a suitable thermal resistance provided by the electronics substrate **140** (e.g., through thickness, material selection, a combination of features) could produce a thermal couple between the heater-sensor die **111** and the heat sink support **151** to permit the heating capacity of the heater-sensor die **111** to achieve the heating times and/or heating ramp rate required by the application, while still allowing adequate cooling rates. Additionally, the second variation can provide increased backside support to each of the set of heater-sensor dies **110** as well as increased surface for adhesion.

In specific examples of the second variation, heat sinking and supporting the "backside" of the electronics substrate **140** can be implemented across multiple heater-sensor dies **111**, separated by Society for Laboratory Automation and Screening (SLAS) standard spacings, such as 9 mm, 4.5 mm or 2.25 mm spacings. The heat sink support **151** material (e.g., aluminum, copper, silver) in the specific examples is mated with the electronics substrate **140** at each heater-sensor die location, with an air gap positioned laterally between each heater-sensor die location. This configuration can further function to reduce cross talk across a set of detection chambers in contact with the set of heater-sensor dies **110**. The set of heat sink supports **150** can, however, be configured in any other suitable manner to provide heat dissipation within the system **100**, without obstruction of optical detection apparatus, and with provision of desired heat ramping and/or cycling behavior.

The set of elastic elements **160** is preferably coupled to a first surface **104a** of the electronics substrate **140**, as shown in FIG. 4A, and functions to promote contact between the set

US 10,239,060 B2

11

of heater-sensor dies **110** and detection chambers (e.g., reaction vessels, detection chambers) for sample processing according to molecular diagnostic protocols. The set of elastic elements **160** can comprise any one more of springs and elastomeric elements, which can deform and provide transmit a biasing force, through the electronics substrate **140**, to reinforce contact between a set of detection chambers and the set of heater-sensor dies **110**. The set of elastic elements **160** can, however, additionally or alternatively include any other suitable elements configured to provide a biasing force that reinforces contact between a set of detection chambers and the set of heater-sensor dies **110** in an elastic or a non-elastic manner. In one such alternative variation, the system **100** can include one or more actuators configured to drive each of the set of heater-sensor dies **111** toward a corresponding detection chamber, and in another such alternative variation, the system **100** can include a set of magnets (e.g., including magnet pairs surrounding the set of heater-sensor dies **110** and a corresponding set of detection chambers), that function to reinforce coupling between the set of heater-sensor dies **110** and the set of detection chambers. However, any other suitable elements can additionally or alternatively be used to facilitate uniform and consistent coupling between the set of heater-sensor dies **110** and a set of detection chambers.

In embodiments of the system **100** including a set of elastic elements **160**, the set of elastic elements **160** is preferably coupled to a first surface **104a** of the electronics substrate **140**, such that each elastic element in the set of elastic elements **160** facilitates contact between a heater-sensor die **111** and a corresponding detection chamber. In a first variation, the set of elastic elements **160** is coupled to first surface **104a** of a flexible PCB of the electronics substrate **140**, as shown in FIG. 4A. In the first variation, contact between each heater-sensor die **111** and a corresponding detection chamber is thus maintained by a biasing force provided by an individual spring through the flexible PCB of the electronics substrate **140**. In the first variation, the number of elastic elements in the set of elastic elements **160** is equal to the number of heater-sensor dies in the set of heater-sensor dies **110**, such that the set of elastic elements **160** and the set of heater-sensor dies **110** are paired in a one-to-one manner. Alternatives to the first variation can, however, comprise any suitable number of elastic elements in relation to a number of heater-sensor dies **110**. In a second variation, the set of heater-sensor dies **110** is coupled to a second surface **104b** of a rigid PCB of the electronics substrate **140**, with the set of elastic elements **160** coupled to the first surface **104a** of the rigid PCB. In the second variation, the set of elastic elements **160** thus functions to collectively transfer a force through the rigid PCB to maintain contact between the set of heater-sensor dies **110** and the detection chambers. Alternatives to the second variation can also comprise any suitable number of springs in relation to a number of heater-sensor dies in the set of heater-sensor dies **110**. Furthermore, variations of the system **100** can include one or more elastic elements coupled to any other elements directly or indirectly coupled to the set of heater-sensor dies **110**. For instance, the system **100** can additionally or alternatively include one or more springs **160'** coupled to base surfaces of the set of heat-sink supports **150** interfacing with the set of heater-sensor dies, in order to transmit biasing forces.

As shown in FIGS. 1A and 1B, the system **100** can further comprise a controller **165**, which functions to automate and/or control heating parameters provided by the set of heater-sensor dies **110**. The controller **165** can further be

12

configured to provide heat parameter output commands to the heating element(s) **114**, and/or configured to receive communication of heating parameters (e.g., detected temperatures) sensed at the sensing element(s) **115** of the system **100**. The controller **165** preferably comprises a proportion-integral-derivative (PID) controller, but can alternatively be any other suitable controller **165**. The controller **165** preferably interfaces with the set of heater-sensor dies **110** through the electronics substrate **140** by a connector; however, the controller **165** can interface with the set of heater-sensor dies **110** in any alternative suitable manner. Preferably, the controller **165** is configured to automate and control heat output parameters, including any one or more of: heating temperatures, heating ramp rates, heating times (e.g., holding times), and any other suitable heating parameter(s). Furthermore, the controller **165** can be configured to control individual heater-sensor dies **111** in order to provide unique heating parameters for individual detection chambers and/or can be configured to provide common heating parameters for all heater-sensor dies **111** in the set of heater-sensor dies **110**. In a specific example, the controller **165** comprises a Yokogawa UT750 PID controller, an Arduino UNO R3 microcontroller configured to cycle the UT750 through temperature stages and to control temperature holding, a resistance-to-voltage conversion circuit, and two power supplies—a first power supply configured to supply power to the set of heater-sensor dies **110** and a second power supply configured to supply voltage to the resistance-to-voltage conversion circuit. In the specific example, the controller **165** comprises a resistance-to-voltage conversion circuit because the UT750 PID controller requires voltage as an input for PID control. In another specific example, the controller **165** comprises a National Instruments LabView based system comprised of an NI cDAQ-9178 chassis with an NI 9219 universal analog input card and an NI 9485 eight-channel solid-state relay sourcing or sinking digital output module solid-state relay card. In this specific example, the cDAQ-9178 supports the NI 9219 and NI 9485 cards, the NI 9219 is used to obtain the RTD inputs, and the NI 9485 cycles the power supply voltage to individual heater-sensor dies of the set of heater-sensor dies **110**. Further, in this specific example, the controller **165** is expandable to 12 or more channels through the use of additional NI 9219 and NI 9485 cards, each of which can handle several channels.

As shown in FIGS. 8A and 8B, the system **100** can further comprise a cooling subsystem **170**, which functions to provide heat transfer from the system **100** in order to further enhance controlled heating and cooling by the system **100**. The cooling subsystem **170** is preferably configured to provide at least one of convective cooling and conductive cooling of the system **100**, but can alternatively be configured to provide any other suitable cooling mechanism or combination of cooling mechanisms. In one variation, the cooling subsystem **170** can comprise a fan **171** that provides convective heat transfer from the system **100**. In this variation, the fan **171** can be coupled to any suitable element of the system **100**, such as the set of heat sink supports **150**, as shown in A. Furthermore, alternatives to this variation can comprise any suitable number of fans of any suitable dimension and configuration, examples of which are shown in FIGS. 7A and 7B. In one such example, the system can include a set of cooling elements integrated with each heat sink support of the set of heat sink supports. In another variation, the cooling subsystem **170** can additionally or alternatively comprise a Peltier device, as shown in FIG. 7C. The Peltier device can be cooled and maintained at a defined

US 10,239,060 B2

13

temperature (e.g., in the 10-25 C range) to provide a substantial temperature gradient for cooling during a thermal cycling process, which can decrease cooling times and/or cycle times. In yet another variation, the cooling subsystem 170 can additionally or alternatively comprise a liquid cooling system (e.g., water cooling system) configured to surround and absorb heat from one or more heater-sensor dies of the set of heater-sensor dies 110, for instance, by way of the set of heat sink supports 150. The cooling subsystem 170 can additionally or alternatively comprise any other suitable cooling element(s).

In some variations, reflection from the set of heater-sensor dies 110 can interfere with light transmitted to photodetectors of an optical subsystem opposed (e.g., directly opposed, in opposition) to the set of heater-sensor dies 110 (e.g., light emitted from the set of biological samples, light transmitted through filters of an optical subsystem), especially in configurations wherein a set of detection chambers is configured between the set of heater-sensor dies and optical elements of an optical subsystem. In these variations, the set of heater-sensor dies 110 can include elements that reduce or eliminate reflection from any portion of the set of heater-sensor dies (e.g., reflection from the heating region, etc.), thereby facilitating analysis of a set of biological samples within the set of detection chambers. In one variation, the set of heater-sensor dies 110 can include or be coupled to one or more non-reflective coatings 180 at surfaces of the set of heater-sensor dies 110 upon which light from the optical subsystem impinges. In a specific example, the non-reflective coating 180 can comprise a high-temperature paint (e.g., dark paint, flat paint) that functions to absorb and/or diffuse light from an opposing optical subsystem, while facilitating heat transfer to a set of detection chambers in thermal communication with the set of heater-sensor dies 110. In another variation, the set of heater-sensor dies 110 can be configured to be in misalignment with photodetectors of the optical subsystem, such that reflection does not interfere with light transmitted to the photodetectors of the optical subsystem. In one example, the set of heater-sensor dies 110 can be configured to heat a set of detection chambers from a first direction, and the optical subsystem can be configured to receive light from the set of detection chambers from a second direction (e.g., a direction non-parallel to the first direction), such that reflection from the set of heater-sensor dies 110 does not cause interference. In still other variations, the set of heater-sensor dies 110 can include any other suitable elements (e.g., coatings, layers, etc.) and/or be configured in any other suitable manner that eliminates, prevents, or mitigates reflection from the set of heater-sensor dies 110 from interfering with light transmitted to photodetectors of an optical subsystem in opposition to the set of heater-sensor dies 110.

Variations of the system 100 can, however, include any other suitable element(s) configured to provide uniform, accurate, precise, and reliable heating of one or more detection chambers in thermal communication with the system 100. Furthermore, as a person skilled in the art will recognize from the previous detailed description and from the figures, modifications and changes can be made to the preferred embodiments of the system 100 without departing from the scope of the system 100.

2. Method of Manufacture

As shown in FIG. 8, a method 200 of manufacturing a system for thermocycling biological sample within detection chambers comprises: forming a first insulating layer coupled to exposed surfaces of a substrate S210; depositing an adhesion material layer of a heating region onto the first

14

insulating layer of the substrate S220; depositing a noble material layer of the heating region onto the adhesion material layer S230; removing material of the heating region S240, thereby forming a pattern of voids that defines a coarse pattern associated with a heating element of the heating region and a fine pattern, integrated into the coarse pattern and associated with a sensing element of the heating region; forming a second insulating layer coupled to the heating region and to the first insulating layer by way of the pattern of voids S250; and annealing the first insulating layer, the heating region, and the second insulating layer for a first duration S260.

The method 200 functions to generate heating and sensing elements of a thermocycling system that can provide rapid thermocycling without significant power requirements, while ensuring a suitably close correlation between an actual heating temperature and a temperature set-point (i.e., an intended heating temperature). In some embodiments, the method 200 can function to produce a heating and sensing element of an integrated heater-sensor die as described in Section 1 above, which can controllably and individually heat small sample volumes (e.g., picoliters, nanoliters, microliters). Furthermore, the method 200 of manufacture preferably implements a priori predictions of electrical resistance values of thin film combinations of the set of heater-sensor dies 110, as described in Section 1 above, and accounts for and/or prevents signal drift in relation to actual and intended heating temperatures, to maintain controlled sample heating.

Block S210 recites: forming a first insulating layer coupled to exposed surfaces of a substrate, which functions to electrically isolate portions of a heating region from other elements of the thermocycling system. The substrate is preferably a silicon substrate, but can alternatively be any other suitable semi-conducting, or non-conducting substrate. As such, in variations, the substrate can be composed of a semi-conducting material (e.g., silicon, quartz, gallium arsenide), and/or an insulating material (e.g., glass, ceramic). In some variations, the substrate 130 can even comprise a combination of materials (e.g., as in a composite, as in an alloy). In examples wherein the substrate is a silicon substrate, the substrate can be composed of silicon with any suitable type (e.g., P-type), doping (e.g., boron-doping), miller index orientation, resistivity, thickness, total thickness variation, and/or polish.

In forming the first insulating layer, Block S210 can be performed using any one or more of: thermal oxide growth, chemical vapor deposition (CVD), spin coating, spray coating, and any other suitable method of depositing a localized layer of an insulating material. Preferably, the first insulating layer is composed of an insulating oxide material, and in examples can include any one or more of: a thermally grown silicon oxide, a chemical vapor deposited oxide, a deposited titanium oxide, a deposited tantalum oxide, and any other suitable oxide grown and/or deposited in any other suitable manner. However, the first insulating layer can additionally or alternatively include an insulating polymer (e.g., a polyimide, a cyanate ester, a bismaleimide, a benzoxazine, a phthalonitrile, a phenolic, etc.) that is chemical and heat resistant and/or any other suitable material (e.g., chemical vapor deposited nitride, other nitride, paralene, etc.) that is configured to provide the first insulating layer.

In one example of Block S210, the first insulating layer comprises an oxide material, and is formed by growing the oxide material on a substrate. In one example of Block S210, the insulating layer comprises a 0.2 mm layer of silicon oxide, and is formed on a 100 mm silicon wafer using

US 10,239,060 B2

15

thermal oxidation at 900° C. using water vapor (i.e., in wet oxidation) or oxygen (i.e., in dry oxidation) as the oxidant. In alternative variations and examples of Block S210, the first insulating layer can be formed using high or low temperature thermal oxidation, using any suitable oxidant, and/or using any other suitable method (e.g., fluid deposition of an electrically insulating polymer, softbaking/hardbaking of a deposited polymer, etc.).

Block S220 recites: depositing an adhesion material layer of a heating region onto the first insulating layer of the substrate, which functions to facilitate bonding of a noble material layer of the heating region to the first insulating layer as in Block S230. The adhesion material preferably comprises an adhesion material that is characterized as active in reacting with the first insulating layer, in order to facilitate coupling between the first insulating layer and the adhesion material. However, the adhesion material of the adhesion material layer can additionally or alternatively have any other suitable characteristic(s). In variations wherein the first insulating layer comprises an insulating oxide, the adhesion materials used in the adhesion material layer(s) can comprise materials that are oxygen-active to chemically react with an oxide surface (e.g., materials that have a high heat of oxide formation). In examples of oxygen-active materials, the adhesion material layer can be composed of any one or more of: chromium, titanium, niobium, vanadium, any other suitable adhesion material that reacts with the insulating layer, and any combination or alloy of any of the above materials.

In Block S220, the adhesion material layer is preferably coupled to the first insulating layer by one or more of evaporation and sputtering. However, in alternative variations, the adhesion material layer can be coupled to the first insulating layer by any one or more of: deposition (e.g., electrodeposition, CVD, etc.), plating (e.g., chemical plating, electroplating), and any other suitable process of coupling the adhesion material layer to the first insulating layer. Furthermore, in examples wherein the adhesion material layer is evaporated or sputtered, the insulating layer-substrate subassembly generated in Block S210 can be translated or rotated in order to facilitate uniform deposition of the adhesion material layer. In Block S220, the adhesion material layer is preferably processed to a thickness of under 100 nm; however, the adhesion material layer can alternatively be processed to any other suitable thickness. In specific examples, the adhesion material layer comprises a 50 nm thick layer of chromium, or a 50 nm thick layer of a combination of titanium and tungsten.

Block S230 recites: depositing a noble material layer of the heating region onto the adhesion material layer, which functions to form a portion of a heating region comprising a heating element and a sensing element of the system. The noble material preferably comprises a noble material that is characterized as having good thermal conductivity and affinity to coupling with the adhesion material layer in a reliable manner during thermocycling of the system. The noble material layer can alternatively have characteristics including any one or more of: characteristics that inhibit diffusion between the adhesion material layer and the noble material layer, high fatigue resistance, high fracture resistance, and any other suitable property that provides reliability during thermocycling of the system. In examples of noble materials that operate well with examples of adhesion materials described above, the noble material layer can be composed of any one or more of: gold, platinum, tungsten, palladium,

16

any other noble material that interacts well with the adhesion material layer, and any combination or alloy of any of the above materials.

In Block S230, the noble material layer is preferably coupled to the adhesion material layer by one or more of evaporation and sputtering. However, in alternative variations, the noble material layer can be coupled to the adhesion material layer by any one or more of: deposition (e.g., electrodeposition, CVD, etc.), plating (e.g., chemical plating, electroplating), and any other suitable process of coupling the noble material layer to the adhesion material layer. Furthermore, in examples wherein the noble material layer is evaporated or sputtered, the adhesion material layer—insulating layer-substrate subassembly generated in Block S220 can be translated or rotated in order to facilitate uniform deposition of the noble material layer. In Block S230, the noble material layer is preferably processed to a thickness of under 500 nm; however, the noble material layer can alternatively be processed to any other suitable thickness. In specific examples, the noble material layer comprises a 200 nm thick layer of gold, a 300 nm thick layer of gold, a 440 nm thick layer of gold, and/or a 100 nm thick layer of platinum.

In some variations, the method 200 can further include Block S225, which recites: depositing an intermediate buffer layer configured between the adhesion material layer and the noble material layer. Block S225 functions to provide a layer that reduces or prevents diffusion between the noble material layer and the adhesion material layer, thus improving dynamic stability (e.g., in electrical resistance behavior) of the system. In variations, the intermediate buffer layer thus has characteristics that contribute to behavior as a barrier against diffusion between the adhesion material layer and the noble material layer. In examples, the intermediate buffer layer comprises platinum and/or titanium, and can be processed onto an adhesion material layer by one or more of evaporation and sputtering. However, in alternative variations, the intermediate buffer layer can be coupled to the adhesion material layer by any one or more of: deposition (e.g., electrodeposition, CVD, etc.), plating (e.g., chemical plating, electroplating), and any other suitable process of coupling the intermediate buffer layer to adhesion material layer, prior to coupling of noble material layer to the intermediate buffer layer. The intermediate buffer layer is preferably processed to a thickness of less than 150 nm, and in specific examples can comprise a 100 nm thick layer of platinum, or a 50 nm thick layer of titanium. However, the intermediate buffer layer can alternatively have any other suitable thickness. Furthermore, variations of the heating region 113 comprising adhesion material layers and noble material layers can include any suitable number of adhesion material layers, noble material layers, and intermediate buffer layers that prevent diffusion between an adhesion material layer into an adjacent noble material layer, wherein each layer can have any other suitable thickness.

Block S240 recites: removing material of the heating region, which functions to form a pattern of voids that defines a coarse pattern associated with a heating element of the heating region and a fine region, integrated into the coarse pattern and associated with a sensing element of the heating region. Block S240 functions to produce elements of the heating region with desired resistance behavior and characteristics, in order to provide reliable and accurate heat parameter sensing and output within the system. Block S240 preferably provides one or more heating elements and sensing elements integrated with the heating elements, wherein the heating element(s) and the sensing element(s)

US 10,239,060 B2

17

have desired theoretical resistance characteristics as described in Section 1.1.1 above; however, Block S240 can alternatively provide heating elements and sensing elements with any other suitable resistance characteristics.

Block S240 preferably produces a pattern of voids having geometric features (e.g., width, thickness, length, spacing) that facilitate uniform heating and provide desired heating and sensing characteristics (e.g., resistance characteristics). In some variations, the pattern of voids can define any one or more of: linear segments, non-linear segments, boustrophedonic segments, continuous segments, non-continuous segments, and any other suitable segment(s) having any other suitable geometry (i.e., width, depth, height, length, path, etc.) within an assembly of the adhesion material layer(s), noble material layer(s), and/or intermediate buffer layer(s) produced in Blocks S220, S230 and/or S225. The pattern of voids produced in Block S240 can further be symmetric about any suitable reference (e.g., reference line, reference plane, etc.), or can alternatively be non-symmetric. Furthermore, in some variations, the pattern of voids can define a global morphology (e.g., circular footprint, ellipsoidal footprint, polygonal footprint, etc.) at a first scale (e.g., macroscopic scale) but have a local morphology at a second scale (e.g., microscopic scale), wherein the local morphology provides desired characteristics (e.g., resistance characteristics) that are attributed to elements (e.g., heating elements, sensing elements) of the heating region. As such, the global morphology can provide conformation (e.g., in shape) between the heating region and a detection chamber configured to contact the heating region, and the local morphology can provide uniform heating and/or accurate sensing of heating parameters by utilizing structural features having a smaller governing dimension.

The pattern of voids is preferably defined entirely through the assembly of the adhesion material layer(s), noble material layer(s), and/or intermediate buffer layer(s) produced in Blocks S220, S230 and/or S225 (e.g., through the assembly to the first insulating layer); however, the pattern of voids can alternatively be defined to any other suitable thickness through the assembly. Preferably, Block S240 includes producing the pattern of voids by way of a lithographic process (e.g., lithographic process with a positive resist, lithographic process with a negative resist). However, producing the pattern of voids in Block S240 can additionally or alternatively be performed using any one or more of: etching, punching, die-cutting, water cutting, laser cutting, and any other suitable method of removing material. In one example, the assembly comprising the adhesion material layer and the noble material layer produced in examples of Blocks S220 and S230 can be covered with positive photoresist (e.g., a photomask designed according to the heating pattern) and lithographically etched in exposed regions. In the example, the positive photoresist can then be removed to reveal the pattern of voids. In other variations of the example, the pattern of voids can be produced using any lithographic method, using positive and/or negative etching to form the heating pattern, and/or using any other suitable method. In one example of an alternative implementation of Step S240, the pattern of voids can be produced using a lift-off process, wherein a sacrificial layer is used to define the pattern of voids, the heating region material(s) is (are) deposited, and then the sacrificial layer is removed to reveal the heating pattern.

In a specific example of Block S240, an assembly comprising an adhesion material layer, a noble material layer, and an intermediate buffer layer configured between the adhesion material layer and the noble material layer is

18

configured to have a global footprint defining circular region having a diameter of 5 mm. The pattern of voids, produced by a lithographic process, defines three heating elements including: a central circular heating element and two circumferential heating elements, as shown in FIG. 3, configured to form a boundary with a serpentine-shaped pattern about the central circular heating element. In the specific example, the two circumferential heating elements are each configured to form a semicircular perimeter about the central circular heating element. The pattern of voids produced in the specific example of Block S240 further comprises three integrated sensing elements (i.e., resistance temperature sensors, RTDs) distributed at three locations within the 5 mm circular footprint. In the specific example, the pattern of voids was designed using a layout editor (e.g., Mentor Graphics™ or L-Edit™), to define the heating elements and the sensing elements according to desired resistance characteristics as described in Section 1.1.1 above. The specific example of Block S240 produced heating elements having coarse patterning, and sensing elements 115 integrated with the heating elements and having fine patterning, as shown in FIGS. 3A and 3B. Variations of the specific example of Block S240 can, however, generate any other suitable pattern of voids to define the heating element(s) and the sensing element(s) in any other suitable manner.

Blocks S210, S220, S230, and S240 are preferably performed in the order described; however, Blocks S210, S220, S230, and S240 can alternatively be performed in any other suitable order. For instance, in one variation the patterned assembly comprising the adhesion material layer(s), the noble material layer(s), and/or the intermediate buffer layer(s) can be formed by any suitable method (e.g., molding, laser cutting, stamping, 3D printing, stereolithography, etc.) and then coupled to the first insulating layer according to any suitable manner.

Block S250 recites: forming a second insulating layer coupled to the heating region and to the first insulating layer by way of the pattern of voids, which functions to electrically isolate portions of a heating region from other elements of the thermocycling system, and to further provide stability within the system. In forming the second insulating layer, Block S250 is preferably performed using a deposition process (e.g., CVD); however, Block S250 can additionally or alternatively be performed using any one or more of: thermal oxide growth, spin coating, spray coating, and any other suitable method of depositing a localized layer of an insulating material. Preferably, the second insulating layer is composed of an insulating oxide material, and in examples can include any one or more of: a silicon oxide (e.g., deposited by CVD, thermally grown), a titanium oxide, a tantalum oxide, and any other suitable oxide grown and/or deposited in any other suitable manner. However, the first second layer can additionally or alternatively include an insulating polymer (e.g., a polyimide, a cyanate ester, a bismaleimide, a benzoxazine, a phthalonitrile, a phenolic, etc.) that is chemical and heat resistant and can be processed onto the first insulating layer and the heating region by any one or more of: casting (e.g., drop casting), printing, dipping, and using any other suitable method. The second insulating layer processed in Block S250 can additionally or alternatively comprise any other suitable material (e.g., chemical vapor deposited nitride, other nitride, paraflex, etc.) that is configured to provide the second insulating layer.

Block S260 recites: annealing the first insulating layer, the heating region, and the second insulating layer for a first duration, which functions to stabilize dynamic resistance behavior of heating and sensing elements of the system.

Since a majority of changes in resistance (e.g., drift) come from thermal reactions, settling, diffusion, and improved adhesion, annealing can accelerate the processes that lead to changes in resistance, thus producing a system with stable resistance behavior (e.g., stable for 3-5 years) by driving dynamic processes toward an equilibrium state (or other stable state) prior to use of the system in sample-processing applications. In Block S260, annealing can comprise a single stage of annealing or multiple stages of annealing, and can additionally or alternatively comprise different conditions (e.g., temperatures, durations, environmental conditions) during the annealing stage(s). Preferably, annealing thus produces stable resistances that are substantially low (in order to enable driving of the system at low voltages) and have limited variability, indicating that underlying material dynamics have reached equilibrium.

In Block S260, annealing is preferably performed at constant temperature, and in variations, can be performed at a temperature between 300 C and 600 C (or any other suitable temperature below a melting temperature of the noble material layer and the adhesion material layer); however, annealing can alternatively be performed at any other suitable temperature or range of temperatures, and can additionally or alternatively be performed at a non-constant temperature (e.g., cyclically varying temperature, non-cyclically varying temperature). Annealing is preferably performed in an inert atmosphere (e.g., inert N₂ atmosphere) to control reactions between the atmosphere and elements being annealed; however, annealing can alternatively be performed in any other suitable atmosphere. In a specific example, Block S240 includes annealing the first insulating layer, the heating region, and the second insulating layer at 400 F in an inert N₂ atmosphere for one hour, which can, in some variations of the specific example, be extended for up to 2 hours following the first hour of annealing.

As shown in FIG. 8, the method 200 can further include Block S270, which recites: coupling a non-reflective coating to at least one heater-sensor die of the set of heater-sensor dies. Block S270 functions to process at least a subset of the set of heater-sensor dies 110 so that they do not interfere with light transmitted to photodetectors of an optical subsystem opposed (e.g., directly opposed, in opposition) to the set of heater-sensor dies 110 (e.g., light emitted from the set of biological samples, light transmitted through filters of an optical subsystem), especially in configurations wherein a set of detection chambers is configured between the set of heater-sensor dies and optical elements of an optical subsystem. The non-reflective coating is preferably coupled identically to all heater-sensor dies of the set of heater sensor dies; however, the non-reflective coating can alternatively be coupled non-identically to one or more heater-sensor dies of the set of heater-sensor dies. As such, in variations, one or more subsets of the set of heater-sensor dies can be coupled to non-reflective coatings in a manner that provides different light reflection properties for the subset(s) of the set of heater-sensor dies.

In Block S270, the non-reflective coating is preferably a material layer that is applied superficial to at least one of the first insulating layer and the second insulating layer processed in variations of Blocks S210 and S250, respectively. In one example, the non-reflective coating processed in Block S270 can comprise a high-temperature paint (e.g., dark paint, flat paint) that functions to absorb and/or diffuse light from an opposing optical subsystem, while facilitating heat transfer to a set of detection chambers in thermal communication with the set of heater-sensor dies. In this example, the high-temperature paint can be applied by any

one or more of: brushing, spraying, dipping, printing, and any other suitable method of coupling the high-temperature paint to one or more surfaces of at least a subset of the set of heater-sensor dies. However, the non-reflective coating can alternatively be processed simultaneously with or can comprise one or more of the first insulating layer and the second insulating layer processed in variations of Blocks S210 and S260, respectively. For instance, one or more of the first and the second insulating layer can include a modified oxide layer that has low-reflectivity, thus preventing interference caused by light reflected from the set of heater-sensor dies. In some extreme variations, however, mitigation of interference due to reflected light from the set of heater-sensor dies can be produced by configuring the set of heater-sensor dies to be in misalignment with photodetectors of the optical subsystem, such that reflection does not interfere with light transmitted to the photodetectors of the optical subsystem, in modified versions of Block S270. For instance, the set of heater-sensor dies can be configured to heat a set of detection chambers from a first direction, and the optical subsystem can be configured to receive light from the set of detection chambers from a second direction (e.g., a direction non-parallel to the first direction), such that reflection from the set of heater-sensor dies 110 does not cause interference. In still other variations of Block S270, the set of heater-sensor dies can be processed with any other suitable elements (e.g., coatings, layers, etc.) and/or be configured in any other suitable manner that eliminates, prevents, or mitigates reflection from the set of heater-sensor dies from interfering with light transmitted to photodetectors of an optical subsystem in opposition to the set of heater-sensor dies 110.

The method 200 can additionally or alternatively include any other suitable blocks or steps configured to facilitate fabrication of a thermocycling element that can heat small volumes (e.g., microliter volumes, nanoliter volumes, picoliter volumes) of biological samples within containers in contact with the thermocycling element. For instance, the method 200 can include any one or more of: coupling the heating region to an electronics substrate S280; coupling the electronics substrate to an elastic element configured to bias the system against a detection chamber S290; and coupling at least one of the electronics substrate and the heating region to a heat sink-support S300 that facilitates heat dissipation within the system, while providing mechanical support for the heating region within the system. The method 200 can, however, include any other suitable blocks or steps.

The system 100 and/or method 200 of the preferred embodiment and variations thereof can be embodied and/or implemented at least in part as a machine configured to receive a computer-readable medium storing computer-readable instructions. The instructions are preferably executed by computer-executable components preferably integrated with the system 300 and one or more portions of the processor 350. The computer-readable medium can be stored on any suitable computer-readable media such as RAMs, ROMs, flash memory, EEPROMs, optical devices (CD or DVD), hard drives, floppy drives, or any suitable device. The computer-executable component is preferably a general or application specific processor, but any suitable dedicated hardware or hardware/firmware combination device can alternatively or additionally execute the instructions.

The FIGURES illustrate the architecture, functionality and operation of possible implementations of methods according to preferred embodiments, example configura-

US 10,239,060 B2

21

tions, and variations thereof. In this regard, each block in the flowchart or block diagrams may represent a module, segment, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that, in some alternative implementations, the functions noted in the block can occur out of the order noted in the FIGURES. For example, two blocks shown in succession may, in fact, be executed substantially concurrently, or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved. It will also be noted that each block of the block diagrams and/or flowchart illustration, and combinations of blocks in the block diagrams and/or flowchart illustration, can be implemented by special purpose hardware-based systems that perform the specified functions or acts, or combinations of special purpose hardware and computer instructions.

As a person skilled in the art will recognize from the previous detailed description and from the figures and claims, modifications and changes can be made to the preferred embodiments of the invention without departing from the scope of this invention defined in the following claims.

We claim:

1. A system for thermocycling biological samples within detection chambers comprising:

a set of heater-sensor dies, each heater-sensor die in the set of heater-sensor dies comprising:

an assembly including a first insulating layer;

a heating region comprising a heating element, a sensing element, an adhesion material layer coupled to the first insulating layer, and a noble metal layer coupled to the adhesion material layer;

a second insulating layer coupled to the first insulating layer through a pattern of voids in the heating region; an electronics substrate configured to couple the heating elements and the sensing elements of the set of heater-sensor dies to a controller; and

a set of elastic elements configured to bias each of the set of heater-sensor dies against a set of detection chambers during operation;

wherein the pattern of voids in the heating region defines:

a first pattern comprising a global morphology at a first size scale the first pattern associated with the heating element of the heating region; and

a second pattern comprising a local morphology at a second size scale smaller than the first size scale, the second pattern integrated into the first pattern and associated with the sensing element of the heating region.

2. The system of claim 1, wherein the set of elastic elements is coupled to a second substrate surface of the electronics substrate opposing a first substrate surface of the electronics substrate that interfaces with the assemblies of the set of heater-sensor dies.

3. The system of claim 2, wherein the electronics substrate is a flexible substrate and wherein the system further comprises a set of heat-sink supports coupled to at least one of the set of heater-sensor dies and the second substrate surface of the electronics substrate and configured to dissipate heat generated by the set of heater-sensor dies, wherein a base surface of each of the set of heat-sink supports is coupled to an elastic element configured to transmit a biasing force through the flexible substrate, thereby maintaining thermal communication between the set of heater-sensor dies and the set of detection chambers in the configuration wherein the

22

set of heater-sensor dies is in thermal communication with the set of detection chambers.

4. The system of claim 1, wherein the adhesion material layer comprises at least one of chromium, titanium, niobium, and vanadium.

5. The system of claim 1, wherein the noble metal layer comprises at least one of gold, platinum, tungsten, and palladium.

6. The system of claim 1, wherein the adhesion material layer and the noble metal layer are annealed layers that provide stabilized resistance behavior and prevent drift in heating and sensing using the heating region.

7. The system of claim 1, wherein the pattern of voids defines a pattern of boustrophedonic segments, wherein the first pattern includes wide segments associated with the heating element, and wherein the second pattern includes segments, surrounded by and narrower than the wide segments, associated with the sensing element of the heating region.

8. The system of claim 1, wherein the assembly further comprises an intermediate buffer layer comprising at least one of platinum and titanium, situated between the adhesion material layer and the noble metal layer, and operable to provide a barrier against diffusion between the adhesion material layer and the noble metal layer.

9. The system of claim 8, wherein the pattern defined through the adhesion material layer and the noble metal layer is operable to provide a resistance change per temperature change greater than 0.1 ohms/ $^{\circ}$ C.

10. The system of claim 1, wherein each of the set of heater-sensor dies includes a coating, proximal the heating surface, configured to mitigate reflection of light from the heating surface toward photodetectors of an optical subsystem during operation.

11. A system for thermocycling biological samples within detection chambers comprising:

a set of heater-sensor dies, each heater-sensor die in the set of heater-sensor dies comprising:

an assembly including a first insulating layer;

a heating region comprising a heating element, a sensing element, an adhesion material layer coupled to the first insulating layer, and a noble metal layer coupled to the adhesion material layer; and

a second insulating layer coupled to the first insulating layer through a pattern of voids in the heating region;

wherein the pattern of voids in the heating region defines: a first pattern comprising a global morphology at a first size scale, the first pattern associated with the heating element of the heating region; and

a second pattern comprising a local morphology at a second size scale smaller than the first size scale, the second pattern integrated into the first pattern and associated with the sensing element of the heating region;

wherein the assembly further comprises an intermediate buffer layer comprising at least one of platinum and titanium, situated between the adhesion material layer and the noble metal layer, and operable to provide a barrier against diffusion between the adhesion material layer and the noble metal layer.

12. The system of claim 11, further including:

an electronics substrate configured to couple the heating elements and the sensing elements of the set of heater-sensor dies to a controller; and

a set of elastic elements configured to bias each of the set of heater-sensor dies against a set of detection chambers during operation.

US 10,239,060 B2

23

13. The system of claim 12, wherein the set of elastic elements is coupled to a second substrate surface of the electronics substrate opposing a first substrate surface of the electronics substrate that interfaces with the assemblies of the set of heater-sensor dies.

14. The system of claim 13, wherein the electronics substrate is a flexible substrate and wherein the system further comprises a set of heat-sink supports coupled to at least one of the set of heater-sensor dies and the second substrate surface of the electronics substrate and configured to dissipate heat generated by the set of heater-sensor dies, wherein a base surface of each of the set of heat-sink supports is coupled to an elastic element configured to transmit a biasing force through the flexible substrate, thereby maintaining thermal communication between the set of heater-sensor dies and the set of detection chambers in the configuration wherein the set of heater-sensor dies is in thermal communication with the set of detection chambers.

15. The system of claim 11, wherein the adhesion material layer comprises at least one of chromium, titanium, niobium, and vanadium.

16. The system of claim 11, wherein the noble metal layer comprises at least one of gold, platinum, tungsten, and palladium.

24

17. The system of claim 11, wherein the adhesion material layer and the noble metal layer are annealed layers that provide stabilized resistance behavior and prevent drift in heating and sensing using the heating region.

18. The system of claim 11, wherein the pattern of voids defines a pattern of boustrophedonic segments, wherein the first pattern includes wide segments associated with the heating element, and wherein the second pattern includes segments, surrounded by and narrower than the wide segments, associated with the sensing element of the heating region.

19. The system of claim 11, wherein the pattern defined through the adhesion material layer and the noble metal layer is operable to provide a resistance change per temperature change greater than 0.1 ohms/ $^{\circ}$ C.

20. The system of claim 11, wherein each of the set of heater-sensor dies includes a coating, proximal the heating surface, configured to mitigate reflection of light from the heating surface toward photodetectors of an optical subsystem during operation.

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